REMARKS

Status of the Claims

Claims 24-46 are pending in the application.

Claims 24-46 have been rejected.

By way of this amendment, new claims 47-56 have been added.

Upon entry of this amendment, claims 24-56 will be pending.

Summary of the Amendment

New claims 47-56 have been added to specifically refer to the amino acid sequence of guanylyl cyclase C that is set forth in the specification. Support for the amendment is found on page 13 lines 28-34 and the sequence listing. No new matter has been added.

Rejection under 35 U.S.C. §112, second paragraph

Claims 24-46 have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as his invention.

It is asserted that claims 24-29 and 36-39, and dependent claims 30-35 and 40-45 are vague and indefinite because claims 24-29 and 38-39 recite the term "guanylyl cyclase C" as the sole means of identifying the polynucleotides of the claimed method. It is asserted that the use of laboratory designations to identify a particular molecule renders the claims indefinite because different laboratories may use the same laboratory designations to define completely distinct molecules. Applicants respectfully disagree.

The test for determining whether a claim is clear and definite is whether one skilled in the art would understand the metes and bounds of the claim. In the instant case, one skilled in the art would, at the time the application was filed, clearly and unambiguously recognize what molecule

is being referred to by the term "human guanylyl cyclase C." The human guanylyl cyclase C gene and protein were well known at the time the application was filed and there was broad recognition and agreement regarding the specific identity of the molecules referred to as human guanylyl cyclase C gene and human guanylyl cyclase C protein. There is no evidence in the record to support the assertion that the term is merely a laboratory designation used by different laboratories to define completely distinct molecules. Rather, there is much evidence that the scientific community was in full agreement as to which molecules were referred to by the designation.

a. de Sauvage F.J., et al. (1991) Primary structure and functional expression of the human receptor for Escherichia coli heat-stable enterotoxin. J Biol Chem. 266(27):17912-8, attached hereto as Exhibit 1, refers to a cellular promoter in human intestine cells which binds to the E coli heat stable enterotoxin STa. The reference refers to the protein as human STa receptor.

b. de Sauvage, F.J. et al. (1992) Precursor structure, expression and tissue distribution of human guanylin. Proc. Natl Acad. Sci USA 89(19): 9089-93, attached hereto as Exhibit 2, refers to a receptor for heat enterotoxin STa on human intestinal cells, including the human cell line T84. The reference refers to the protein as STaR or STa receptor.

c. Vaandrager A.B., et al. (1993) Guanylyl cyclase C is an N-linked glycoprotein receptor that accounts for multiple heat-stable enterotoxin-binding proteins in the intestine. J Biol Chem. 268(3):2174-9, attached hereto as Exhibit 3, refers to guanylyl cyclase C and states on page 2174 that intestinal guanylyl cyclase C is STa receptor. References 9-10 are cited in support of this statement. Reference 10 is the de Sauvage paper discussed above (Exhibit 1).a cellular promoter in human intestine cells which binds to the E coli heat stable enterotoxin STa. The reference refers to the protein as human STa receptor.

d. Almenoff, J.S., et al. (1994) Induction of Heat-stable Enterotoxin Receptor Activity by Human Alu Repeat. J Biol Chem 269(24):16410-17, attached hereto as Exhibit 4, has

been cited in the Official Action. Applicant notes that refers to guanylyl cyclase C as an ST receptor which has been cloned. References 8-9 are cited in support of this statement. Reference 9 is the de Sauvage paper discussed above (Exhibit 1).a cellular promoter in human intestine cells which binds to the E coli heat stable enterotoxin STa. The reference refers to the protein as human STa receptor.

e. Krause G. et al., (1994) Distribution of membrane bound guanylyl cyclases in human intestine. Gut. 35(9):1250-7, attached hereto as Exhibit 5, refers to guanylyl cyclase C as a receptor for E. coli heat stable enterotoxin STa and guanylin. In a passage on page 1255, the authors refer to guanylyl cyclase C and cite reference 29 which is the Vaandrager paper discussed above (Exhibit 3).

f. Cetin Y., et al. Enterochromaffin cells of the digestive system: cellular source of guanylin, a guanylate cyclase-activating peptide. (1994) Proc Natl Acad Sci U S A. 91(8):2935-9, attached hereto as Exhibit 6, refers to guanylate cyclase C as a receptor for E. coli heat stable enterotoxin STa and guanylin, referring to references 4 and 5 in support of the statement. Reference 5 is the Vaandrager paper discussed above (Exhibit 3).

g. Rudner X.L., et al. (1995) Regulation of cell signaling by the cytoplasmic domains of the heat-stable enterotoxin receptor: identification of autoinhibitory and activating motifs. Proc Natl Acad Sci U S A. 92(11):5169-73, attached hereto as Exhibit 7, refers to guanylate cyclase C as also being known as StaR, a receptor that E. coli heat stable enterotoxin ST binds to. In referring to the receptor's activity, the authors cite to references 2-4. Reference 2 is the de Sauvage paper discussed above (Exhibit 1).

The seven references provided herein are a sample of the numerous published papers in the scientific literature which clearly reflect the consensus of the subject molecule. One skilled in the art, reviewing these references would clearly conclude that human guanylyl cyclase C, human guanylate cyclase C and STa receptors all refer to the same intestinal receptor. The meaning of

the terms as used by Applicant is unambiguous to those skilled in the art. the evidence of record is overwhelming in this regard.

The Official Action notes Applicant's position that the instant specification discloses that the terms "ST receptor" and "**guanylin** cyclase C" are interchangeable and are broadly meant to refer to receptors found on colorectal cancer cells which bind to ST. The Official Action asserts that Almenoff et al clearly demonstrate that a protein referred to as guanylyl cyclase C is a species of ST receptor. Applicant respectfully urges that while Almenoff et al. report a cDNA which induced expression of an ST-binding phenotype, Almenoff et al. clearly and unambiguously support Applicants position that those skilled in the art would clearly recognize the meaning of the term guanylate cyclase C and the metes and bounds of the claims. As for the issue of whether the terms are interchangeable, Applicant suggests that issue is more properly addressed in the written description and new matter rejections. The rejection under the second paragraph of section 112 relates to whether or not one skilled in the art could clearly and definitely recognize the subject matter claimed by Applicant. The pending claims fully and clearly meet this requirement.

Claims 26, 27, 36, and 37 are rejected for reciting "the extracellular domain of the human guanylyl cyclase C protein". It is asserted that it is unclear what is meant by "guanylyl cyclase C" and this it is asserted that it is unclear what part of guanylyl cyclase C would be the extracellular domain of human guanylyl cyclase C. it is also asserted in the Official Action that the specification does not disclose which region of human guanylyl cyclase C is an extracellular domain. Applicants respectfully disagree.

As discussed above, one skilled in the art would clearly recognize the molecule referred to in the claims as "guanylyl cyclase C." The de Sauvage paper (Exhibit 1) provides a complete description of the structure of the receptor. The Krause paper (Exhibit 5) and the Rudner paper

(Exhibit 7) clearly describe the extracellular domain of GCC. It is well settled that patents need not include, and preferably omit that which is known in the art. Those skilled in the art would clearly recognize what is meant by "guanylyl cyclase C" and its extracellular domain.

The claims are clear and definite. Applicant respectfully requests that the rejection of claims 24-46 under 35 U.S.C!. §112, second paragraph, be withdrawn.

Rejection under 35 U.S.C. §112, first paragraph

Written Description Rejection

Claims 24-46 have been rejected under 35 U.S.C § 112, first paragraph, as failing to comply with the written description requirement. It is asserted that the claims contain subject matter which was not described in the specification in such a way to reasonably convey to those skilled in the art that the inventor, at the time of the application was filed, had possession of the claimed invention. It is asserted that the claims are inclusive of: (1) a genus of polynucleotides encoding human guanylyl cyclase C protein and (2) a genus of polynucleotides encoding the extracellular domain of human guanylyl cyclase C. It is asserted that the specification does not disclose the term "guanylyl cyclase C". Further, It is asserted that the specification does not disclose "the extracellular domain of human guanylyl cyclase C". Applicants respectfully disagree.

Applicant's amendment filed February 27, 2007, amended the claims to refer to "guanylyl cyclase C" instead of "ST receptor". Applicant urges that those of skill in the art would recognize that the two terms are interchangeable. The instant specification discloses the terms "ST receptor" and "**guanylin** cyclase C" as being are interchangeable and meant to refer to receptors found on colorectal cancer ceils which bind to ST. Applicant urges that one skilled in the art, i.e. someone familiar with the prior art, reviewing the specification in its entirety, would

clearly conclude that the term "ST receptor" specifically refers to "guanylyl cyclase C." As noted above, one skilled in the art would immediately recognize what is meant by the term "guanylyl cyclase C" as it appears in the claims. One skilled in the art would know that such receptors bind to guanylin, bind to E. coli heat stable enterotoxin ST, and are alternatively referred to in the literature as "STa receptors". One skilled in the art, familiar with the prior art and reading Applicant's specification could not reasonably conclude that ST receptors as disclosed in the specification are a broad genus that includes guanylyl cyclase C as argued in the Official Action. Such a conclusion is not reasonable and not supported by the facts.

Applicant notes that on page 16, lines 8-10, of the specification, Applicant refers to the Rudner paper (Exhibit 7) as describing "the cloning and expression of the extracellular domain of human ST receptor." The Rudner paper clearly refers to the cloning and expression of the extracellular domain of human guanylyl cyclase C, and refers to it as both human guanylyl cyclase C, as well as an alternative designation, STaR, citing the de Sauvage paper (Exhibit 1). Clearly, the specification is not referring to a genus of ST receptors of which guanylyl cyclase C is a member. Rather, it is clear that the terms are all intended to refer to the same molecule.

On page 7, lines 9-11, Applicant incorporated into the specification by reference the disclosure of U.S. patent application no. 08/141,892 which issued as U.S. Patent No. 5,518,888, which is attached hereto as Exhibit 8. Column 3, liners 60-67 of U.S. Patent No. 5,518,888 state:

> As used herein, the term "ST receptor" is meant to refer to the receptors found on colorectal cells, including local and metastasized colorectal cancer cells, which bind to ST. In normal individuals, ST receptors are found exclusively in cells of intestine, in particular in cells in the duodenum, small intestine (jejunum and ileum), the large intestine, colon (cecum, ascending colon, transverse colon, descending colon and sigmoid colon) and rectum.

Column 5, line 56 to column 6, line 32 of U.S. Patent No. 5,518,888 state:

> ST, which is produced by E. coli, as well as other organisms, is responsible for endemic diarrhea in developing countries and travelers diarrhea. ST induces intestinal secretion by binding to specific receptors, ST receptors, in the apical brush border membranes of the mucosal cells lining the intestinal tract. Binding of ST to ST receptors is non-covalent and occurs in a concentration-dependent and saturable fashion. Once bound, ST-ST receptor complexes appear to be internalized by intestinal cells, i.e. transported from the surface into the interior of the cell. Binding of ST to ST receptors triggers a cascade of biochemical reactions in the apical membrane of these cells resulting in the production of a signal which induces intestinal cells to secrete fluids and electrolytes, resulting in diarrhea.
>
> ST receptors are unique in that they are only localized in the apical brush border membranes of the cells lining the intestinal tract. Indeed, they are not found in any other cell type in placental mammals. In addition, ST receptors are almost exclusively localized to the apical membranes, with little being found in the basolateral membranes on the sides of intestinal cells.
>
> Mucosal cells lining the intestine are joined together by tight junctions which form a barrier against the passage of intestinal contents into the blood stream and components of the blood stream into the intestinal lumen. Therefore, the apical location of ST receptors isolates these receptors from the circulatory system so that they may be considered to exist separate from the rest of the body; essentially the "outside" of the body. Therefore, the rest of the body is considered "outside" the intestinal tract.

> Compositions administered "outside" the intestinal tract are maintained apart and segregated from the only cells which normally express ST receptors.
>
> In individuals suffering from colorectal cancer, the cancer cells are often derived from cells that produce and display the ST receptor and these cancer cells continue to produce and display the ST receptor on their cell surfaces. Indeed, T84 cells, which are human colonic adenocarcinoma cells isolated from lung metastases, express ST receptors on their cell surface. Similarly, HT29glu-cells, which are human colonic adenocarcinoma cells, express receptors for ST.

The de Sauvage paper (Exhibit 1) describes expression of STaR in normal intestine. The de Sauvage paper (Exhibit 2) describes expression of STaR in T84 cells. The Krause paper (Exhibit 5), on page 1250 and in the discussion, describes expression of guanylyl cyclase C in normal intestine and T84 cells. Clearly, the ST receptors referred to in the instant specification are guanylyl cyclase C, not a genus of which guanylyl cyclase C is a member.

Almenoff et al is cited in the specification as clearly demonstrating that a protein referred to as guanylyl cyclase C is a species of ST receptor. On the contrary, while Almenoff does disclose the cloning of a cDNA in an SV40 vector which, when expressed in COS cells, induces expression of an ST binding phenotype. That is, Almenoff et al. suggest that the cDNA does not encode a protein that binds to ST but rather, the presence of the protein induces expression of another protein which exhibits ST binding activity. The identity of this another protein whose expression is induced by Almenoff's cDNA clone is not disclosed. Almenoff does not demonstrate that a protein referred to as guanylyl cyclase C is a species of ST receptor at least as the term ST receptor is used in the instant specification and claims as filed. The record clearly supports the conclusion that as used in the instant specification and claims as filed the term ST receptor and guanylyl cyclase C are synonymous and one skilled in the art would clearly

recognize this to be the case. One skilled in the art would clearly recognize that, at the time of the application was filed, Applicant had possession of the claimed invention. One skilled in the art would clearly recognize that, at the time of the application was filed, Applicant was in possession of the claims subject matter referred to as human guanylyl cyclase C protein the extracellular domain of human guanylyl cyclase C. One skilled in the art would recognize that the specification specifically discloses the subject referred to in the current claims as "guanylyl cyclase C" and as "the extracellular domain of human guanylyl cyclase C".

Claims 24-46 are in compliance with the written description requirement of the first paragraph of section 112. Applicants respectfully request that the rejection of clams 24-46 under 35 U.S.C § 112, first paragraph, as failing to comply with the written description requirement be withdrawn.

Enablement Rejection

Claims 24-46 have been rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. It is asserted that the claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Applicants respectfully disagree.

It is asserted that the specification lacks working examples using a vaccine comprising a nucleic acid molecule encoding at least one epitope of human guanylyl cyclase C or human ST receptor protein to treat any individual. It is asserted that the specification does not provide a written description of methods involving polynucleotides encoding "human guanylyl cyclase C" or polynucleotides encoding the extracellular domain of "human guanylyl cyclase C". It is asserted that therapeutic treatments, in general, are unpredictable. Citing Crystal, R.G. (Science, Vol. 270, October 1995, pages 404-410), it is asserted that the gene therapy against tumors is

highly unpredictable and, citing Tait *et al.* (Clin.Cane.Res., Vol. 5, July 1999, pages 1708-1714) that gene therapy is unpredictable in the clinical setting. Moreover, Gura (Science, 1997, 278:1041-1042) is cited to support the assertion that therapeutic cancer treatments, in general, are unpredictable. It is noted that in view of *In re Brana*, the Examiner asserts that successful use of *in vivo* mouse models of colon cancer enables compositions for specific therapeutic effects in humans and does not require human clinical testing.

It is well settled that working examples are not absolutely required. Moreover, as discussed in detail above, the specification does refer to the claimed subject matter, i.e. human guanylyl cyclase C and the extracellular domain of human guanylyl cyclase C. Thus, the specification does provide a written description of methods involving polynucleotides encoding "human guanylyl cyclase C" or polynucleotides encoding the extracellular domain of "human guanylyl cyclase C". Applicants respectfully urge that one skilled in the art would conclude that the specification enables the claimed invention.

Applicant provides herewith an unexecuted declaration of inventor Scott A. Waldman. The unexecuted declaration submitted herewith refers to a published scientific article as well as a manuscript submitted for publication. The disclosure and data in these documents make clear that the claimed invention is operable *in vivo*. The evidence is sufficient to support a finding that one skilled in the art would conclude that the claimed invention is enabled. In particular, the data in Figure 3 of the Snook et al. 2007 paper (Exhibit A of the Unexecuted Declaration submitted herewith) show that immunization protected against lung metastasis in animals challenged with GCC-expressing colorectal cancer cells. The data in the Snook et al. submitted manuscript (Exhibit B of the Unexecuted Declaration submitted herewith) show immunization with GCC-expressing viral vectors opposed the formation of nascent metastases to liver and extended the median survival of mice with established lung metastases following therapeutic immunization.

Antitumor efficacy reflected asymmetric tolerance that was characterized by CD8+ T cell, but not CD4+ T cell or antibody, responses.

The claimed invention is enabled by the specification. The claims are in compliance with the enablement requirement of the first paragraph of 35 U.S.C. § 112. Applicant respectfully requests that the rejection of claims 24-46 under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement, be withdrawn.

New Matter

Claims 24-48 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. It is asserted that the claims contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. It is asserted that the claims contain new matter. Applicant respectfully disagrees.

It is asserted that claims 24-48 recite methods comprising using polynucleotides encoding human guanylyl cyclase C and method using polynucleotides encoding the extracellular domain of human guanylyl cyclase C and that human guanylyl cyclase C and the extracellular domain of human guanylyl cyclase C are not disclosed. It is asserted that contrary to Applicant's assertion, references in the specification to ST receptor are not synonymous with human guanylyl cyclase C.

As set forth above in the section entitled "Written Description," Applicant respectfully urges that, when the facts are viewed in their entirety, one skilled in the art would clearly conclude that Applicant's reference to human ST receptor in the specification is a specific reference to human guanylyl cyclase C. While "guanylyl" is misspelled "guanylin" in the passage referring to the interchangeability of the terms, in the full context of the disclosure, one skilled in the art, who would be familiar with all of the art, would clearly recognize that in view

of Applicant's specification, it is reasonable conclusion and supported by overwhelming evidence to conclude that Applicant's use of the term "ST receptor" referred specifically to guanylyl cyclase C, not a genus that includes guanylyl cyclase C. In the context of the specific disclosure in the specification and the prior art, reference to guanylyl cyclase C and to the extracellular domain of guanylyl cyclase C is not new matter.

Claims 24-48 are in compliance with the requirements of the first paragraph of section 112. The claims do not contain new matter. Applicants respectfully request that the rejection of claims 24-48 under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement, i.e. containing new matter, be withdrawn.

Information Disclosure

As noted in the Official Action, the Declaration of the inventor, Dr. Scott Waldman, submitted with the Amendment filed February 27, 2007, was unexecuted. Applicant's undersigned representative intended to do so shortly after filing the amendment. Through error without deceptive intent, Applicant's undersigned representative did not request an executed copy of the declaration submitted on February 27, 2007, from the inventor. Only upon receiving the Official Action dated May 2, 2007 did Applicant's undersigned representative realize the omission.

The data in the Declaration submitted February 27, 2007, of the inventor, Dr. Scott Waldman, was provided to Applicant's undersigned representative prior to February 27, 2007. Applicant's undersigned representative prepared the declaration submitted with the Amendment filed February 27, 2007, based upon the data provided. On February 27, 2007, Applicant's undersigned representative filed the unexecuted declaration with the Amendment in order to expedite filing. On March 1, 2007, Applicant's undersigned representative joined Pepper Hamilton LLP and, per Applicant's instructions, the present application was transferred to Pepper

Hamilton LLP so that Applicant's undersigned representative could continue being the attorney of record. During this period, Applicant's undersigned representative did not request an executed copy of the declaration submitted February 7, 2007, from the inventor. Upon receipt of the Official Action dated May 2, 2007, Applicant's undersigned representative became aware of the omission.

In view of the Official Action, Applicant's undersigned representative inquired with the Applicant regarding any additional data. Applicant advised Applicant's undersigned representative that more experiments were being planned and performed. Such additional experiments resulted in data which is included in the submitted manuscript (Unexecuted Declaration submitted herewith of Scott A. Waldman, Exhibit B). During Applicant's undersigned representative's preparation of this amendment and the Unexecuted Declaration of Scott A. Waldman submitted herewith, Applicant's undersigned representative requested that the Applicant execute the previously filed Declaration. Applicant advised Applicant's undersigned representative that certain data in the Unexecuted Declaration submitted February 7, 2007, was no longer considered reliable. Specifically, data related to CD4 responses from experiments using immunizations with GST-GCC fusion proteins were concluded not to be reproducible. The data from experiments using viral vectors to deliver nucleotides that encode GCC was found to be reliable. In view of this discovery, Applicant's undersigned representative is providing this explanation. The original omission of providing an executed declaration after initially filing an unexecuted declaration on February 7, 2007, was done in error without deceptive intent. Applicant was unaware that the unexecuted Declaration had been filed February 7, 2007. Applicant's undersigned representative was unaware of the status of the data.

Conclusion

Claims 24-56 are in condition for allowance. The examination of claims 24-56 and passage to allowance are respectfully requested. An early Notice of Allowance is therefore earnestly solicited. Applicants invite the Examiner to contact the undersigned attorney at 215.665.5592 to clarify any unresolved issues raised by this response.

As indicated on the transmittal accompanying this response, the Commissioner is hereby authorized to charge any debit or credit any overpayment to Deposit Account No. 50-0436.

Respectfully submitted,

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Dated: May 2, 2008
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Attachments:

Exhibit 1. de Sauvage F.J., et al. (1991) Primary structure and functional expression of the human receptor for Escherichia coli heat-stable enterotoxin. J Biol Chem. 266(27):17912-8

Exhibit 2. de Sauvage, F.J. et al. (1992) Precursor structure, expression and tissue distribution of human guanylin. Proc. Natl Acad. Sci USA 89(19): 9089-93,

Exhibit 3. Vaandrager A.B., et al. (1993) Guanylyl cyclase C is an N-linked glycoprotein receptor that accounts for multiple heat-stable enterotoxin-binding proteins in the intestine. J Biol Chem. 268(3):2174-9

Exhibit 4. Almenoff, J.S., et al. (1994) Induction of Heat-stable Enterotoxin Receptor Activity by Human Alu Repeat. J Biol Chem 269(24):16410-17

Exhibit 5. Krause G. et al., (1994) Distribution of membrane bound guanylyl cyclases in human intestine. Gut.35(9):1250-7

Exhibit 6. Cetin Y., et al. Enterochromaffin cells of the digestive system: cellular source of guanylin, a guanylate cyclase-activating peptide. (1994) Proc Natl Acad Sci U S A. 91(8):2935-9

Exhibit 7. Rudner X.L., et al. (1995) Regulation of cell signaling by the cytoplasmic domains of the heat-stable enterotoxin receptor: identification of autoinhibitory and activating motifs. Proc Natl Acad Sci U S A. 92(11):5169-73

Exhibit 8. U.S. Patent No. 5,518,888, which is attached hereto as Exhibit 8.

Unexecuted Declaration of Scott A. Waldman including Exhibits A and B

Exhibit A - Snook, A.E. *et al.* Cancer mucosa antigens as a novel immunotherapeutic class of tumor-associated antigen (2007) Clin Pharmacol Ther. 82(6):734-739

Exhibit B. - Snook, A.E. *et al.* Guanylyl cyclase C -induced immunotherapeutic responses opposing tumor metastases without autoimmunity (2008) *Submitted*

EXHIBIT 1

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Printed in U.S.A.

Primary Structure and Functional Expression of the Human Receptor for *Escherichia coli* Heat-stable Enterotoxin*

(Received for publication, April 8, 1991)

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Heat-stable enterotoxin (STa) produced by *Escherichia coli* induces intestinal secretion in mammals by binding to the brush border membrane of the small intestine and activating guanylyl cyclase. We report here the cloning and expression of a cDNA encoding the human receptor for STa. The receptor contains both an extracellular ligand binding site and a cytoplasmic guanylyl cyclase catalytic domain, making it a member of the same receptor family as the natriuretic peptide receptors. Stable mammalian cell lines overexpressing the STa receptor specifically bind ^{125}I-STa (K_d ~1.0 nM) and respond to STa by dramatically increasing (~50-fold) cellular cGMP levels. Sequence comparisons between the human and the rat STa receptors show less conservation in the extracellular domain than similar comparisons of natriuretic peptide receptors. This divergence may indicate important species differences in ligand-receptor interaction.

Escherichia coli heat-stable enterotoxins (STa)[1] are a family of homologous peptides that can cause various diarrheal diseases, including traveler's diarrhea and epidemic diarrhea in newborns (1, 2), accounting for as much as 50% of infant mortality in underdeveloped countries (3). STa has been shown to bind specific high affinity receptors located on the brush border membrane of the small intestine (4, 5). Interaction of STa with its receptor is reported to stimulate guanylyl cyclase activity (6–8). The subsequent increase in intracellular cyclic GMP (cGMP) induces fluid secretion primarily by impairing sodium and chloride absorption across the brush border membrane while stimulating the secretion of chloride and water (6, 9, 10).

Although the biological processes that follow the binding of STa are poorly understood, it has been proposed that the STa receptor and the guanylyl cyclase are two different proteins that are coupled by cytoskeletal components (11, 12). However, the concept of receptor-guanylyl cyclases has emerged recently with the cloning and expression of cDNAs encoding cell surface receptors for the atrial natriuretic peptides (13–16). These studies have demonstrated that the binding sites for the hormones and the guanylyl cyclase catalytic activity reside on the same protein. The two identified natriuretic peptide receptor guanylyl cyclases (ANPRA and ANPRB) have a common architecture. A single transmembrane domain separates the extracellular or binding portion of the receptors from the cytoplasmic domains which can be divided in two parts of approximately equal size. Immediately adjacent to the transmembrane domain is the kinase homology domain that displays sequence homology with the catalytic domains of protein kinases (17). The carboxyl termini of the two natriuretic peptide receptors contain the guanylyl cyclase catalytic activity based on their homology to the soluble guanylyl cyclases (14, 15) and on detection of guanylyl cyclase activity in extracts from bacteria producing the carboxy terminal domain of ANPRA (18).

Recently, using low stringency polymerase chain reaction conditions with degenerate oligonucleotides designed from a highly conserved region within the guanylyl cyclase domains of the natriuretic peptide receptors, Schulz and collaborators (19) have identified a new membrane bound guanylyl cyclase in the rat. This intestinal-specific receptor which is not homologous to the natriuretic peptide receptors in the extracellular domain, was identified as the rat STa receptor. In this paper we report the characterization of the cDNA encoding the human receptor for the *E. coli* STa enterotoxin (STaR). Cells overexpressing STaR bind STa with high affinity, show marked elevation of intracellular cGMP content in response to STa and provide an important model system to study the molecular mechanism of action of STa.

MATERIALS AND METHODS

Isolation and Sequencing of Human STa Receptor cDNA—Total RNA was isolated from a surgical specimen of human ileum using RNAzol (Cinn/Biotex). Five μg of RNA was reverse-transcribed using 1000 units of avian myeloblastosis virus reverse transcriptase (United States Biochemical Corp.) and 5 μg of oligo(dT) as primer. Ten percent of the resulting cDNA was used as template for a polymerase chain reaction containing 10 mM Tris-Cl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each deoxynucleotide, and 0.2 μM of two degenerate primers: antisense 5′GCTAAAAATAGGTGCTATCG-TGGTC(T/C)G(A/T)(C/A)TT3′ and sense 5′TGTGGGAGGAGGA-TCCAGAAAAG(A/C)(G/A)GCC3′, synthesized based on the sequence of the rat STaR (19). The product of the reaction, a 1-kilobase pair fragment corresponding to the 3′ end of the coding region of the rat STaR sequence (amino acids 706–1052), was gel-purified, labeled to high specific activity by random priming, and used to screen 10^7 clones of a human terminal ileum cDNA library (provided by John McLean, Genentech, Inc.) in λgt10. Duplicate filters were hybridized under high stringency conditions at 42 °C in 50% formamide, 5 × SSC, 10 × Denhardt's, 0.05 M sodium phosphate (pH 6.5), 0.1% sodium pyrophosphate, 50 μg/ml of sonicated salmon sperm DNA, and 10% dextran sulfate. Filters were rinsed in 2 × SSC at 42 °C and then washed twice in 0.2 × SSC, 0.1% sodium dodecyl sulfate at 42 °C. Hybridizing phage were plaque-purified, and the largest cDNA inserts were subcloned into the Bluescript plasmid (Stratagene). Both strands were sequenced by the dideoxy chain termination procedure

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank℠/EMBL Data Bank with accession number(s) M73489.

‡ To whom correspondence should be addressed.

[1] The abbreviations used are: STa, heat-stable enterotoxin; ANPR, atrial natriuretic peptide receptor; EBV, Epstein Barr virus; DMEM, Dulbecco's modified Eagle's medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(20). Sequence homology alignments were performed by the method of Hein (21).

STa Receptor Expression—A full-length cDNA fragment, from the 5′ *Xho*I cloning linker to *Hin*dIII (position 3344, Fig. 1), was subcloned into the plasmid pNebo, an Epstein-Barr virus (EBV)-based vector (22) containing a resistance marker for neomycin, the EBV origin of replication, and the EBV nuclear antigen gene EBNA-1.[2] The cDNA insert is under the control of the cytomegalovirus immediate early promoter.

Human embryonic kidney 293 cells maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with F-12 nutrient mixture, 20 mM Hepes (pH 7.4), and 10% fetal bovine serum were transfected by the calcium phosphate method as described (23). Selection for neomycin-resistant cells was begun 48 h after transfection with Geneticin (GIBCO) at 450 μg/ml. Ten days later, resistant colonies were pooled and kept under selection until analysis.

cGMP Assay—Cells expressing the STaR were plated in 6-well dishes (4×10^5 cells/well). Forty-eight hours later, cells were washed once with DMEM containing 20 mM Hepes (pH 7.2), preincubated with DMEM containing 20 mM Hepes, 0.1 mM isobutylmethylxanthine for 10 min at 37 °C, and incubated for 10 min at 37 °C in DMEM containing 20 mM Hepes, 0.1 mM isobutylmethylxanthine, and various concentrations of *E. coli* STa (Sigma). The incubation media was aspirated and replaced with 1 ml of 10% trichloroacetic acid, and the cells were frozen quickly on dry ice. After the samples thawed at room temperature, the cell debris was removed by centrifugation at 2500 × *g* for 10 min. Samples were extracted three times with 0.5 ml of water-saturated ether and warmed to 55 °C for 20 min to evaporate the residual ether. Aliquots were acetylated and analyzed for cGMP concentration by radioimmunoassay according to the manufacturer's instructions (Biomedical Technologies Inc., Stoughton, MA).

^{125}I-STa Binding Assay—STa was radioiodinated to a specific activity of ~2000 Ci/mmol using $Na^{125}I$ and lactoperoxidase (24). [^{125}I-Tyr]STa was purified by reverse phase high performance liquid chromatography as described (25). Transfected cells were washed 3 times with PBS, harvested with PBS + 1 mM EDTA and washed with PBS containing 0.1% bovine serum albumin, 0.02% sodium azide (PBSA buffer). Duplicate samples of 5×10^5 cells in 0.5 ml of PBSA were incubated for 2 h at room temperature while shaking with 50 pM ^{125}I-STa plus various concentrations of unlabeled STa. The assay was terminated by rapid filtration through Whatman GF/C filters previously treated with 1% polyethyleneimine. Filters were rinsed three times with 1 ml of PBS and counted in a γ spectrometer (Iso-Data 100).

RESULTS

STa Receptor cDNA Cloning—Degenerate oligonucleotides corresponding to two regions of the rat STa receptor cDNA (19) were synthesized and used to amplify human ileum cDNA by polymerase chain reaction. A 1-kilobase pair fragment representing the 3′ end of the human STaR coding sequence was isolated and used as an homologous probe to screen a human terminal ileum cDNA library of 10^6 clones. Thirty-four positive phage plaques were obtained and the clone (No. 10) with the largest insert was used for nucleotide sequence determination (Fig. 1). The cDNA covers 3745 base pairs and encompasses the entire coding region for human STaR.

An ATG codon within a favorable consensus sequence for eukaryotic translation initiation (26) is present at nucleotide 49–51 and defines the beginning of an open reading frame of 1073 amino acids. The hydrophobic amino-terminal residues probably constitute a signal peptide which, according to the consensus rules of von Heijne (27), is probably cleaved after residue 23. This would generate a mature protein of 1050 residues with a predicted M_r of 120,707. The open reading frame is terminated by a TAA stop codon followed by 475 base pairs of 3′-untranslated sequence. A consensus polyadenylation signal AAUAAA (28) is present at position 3731. Hydrophobicity analysis predicts a single transmembrane domain from residue 408 to 431 that is followed by the stop transfer sequence Arg-Lys (29). The 407-amino acid extracellular domain contains nine potential *N*-linked glycosylation sites and 8 cysteines. The cytoplasmic domain is 620 amino acids long and contains 10 cysteine residues.

Sequence Comparisons—The overall amino acid sequence identity between the human STaR and the rat STaR is 81%, but this homology is not evenly distributed (Fig. 2). The 620 amino acid cytoplasmic regions are 91% identical, whereas the extracellular domains are 71% identical. Seven out of nine potential *N*-linked glycosylation sites are conserved and all 8 cysteine residues of the human extracellular domain are also present in the rat sequence. The other members of the guanylyl cyclase receptor family, the human atrial natriuretic peptide receptors A (ANPRA, Ref. 15) and B (ANPRB, Ref. 13) (Fig. 3) have three distinct domains that are defined on the basis of amino acid sequence homology. These are the extracellular ligand binding domain and the intracellular kinase homology domain and guanylyl cyclase catalytic domain. The extracellular domain of the human STaR is only 17 and 15% homologous to ANPRA and ANPRB, respectively, whereas ANPRA and ANPRB are 43% identical. Two potential *N*-glycosylation sites and 4 cysteines are conserved between STaR and ANPRA. One potential *N*-glycosylation site and 3 cysteines are conserved between STaR and ANPRB.

In the intracellular region, the kinase homology domain of the STaR is 37 and 36% identical to the equivalent domains of ANPRA and ANPRB, respectively. Notably, as seen with the rat STaR, the consensus G*X*G*X*N nucleotide binding motif present in protein kinases and ANPRA (17) is not conserved. The guanylyl cyclase catalytic domain has 53% identity with both ANPRA and ANPRB catalytic domains.

Expression and Characterization of STaR—To confirm that our cDNA clone encoded the human STa receptor, a cDNA fragment containing the entire open reading frame was inserted under the transcriptional control of the cytomegalovirus immediate-early promoter in the EBV-based plasmid pNebo. The pNebo-STaR expression vector was stably transfected into the human embryonic kidney 293 cell line. Neomycin-resistant transfectants were incubated in the presence of increasing concentrations of *E. coli* STa for 10 min and the intracellular content of cGMP determined (Fig. 4). In unstimulated cells, 0.25 pmol of cGMP/10^6 cells was detected. When stimulated with 5 μM of STa, the cGMP content increased 48-fold to 12 pmol/10^6 cells. Half-maximal accumulation (EC_{50}) of intracellular cGMP is seen at a concentration of 150 nM STa. No elevation of the intracellular content of cGMP levels was observed when these STaR-expressing cells were stimulated with the natriuretic peptides ANP, brain natriuretic peptide, or C-type natriuretic peptide. In addition, STa stimulates only cells expressing the STaR and not cells expressing ANPRA or ANPRB (data not shown).

Specific binding of ^{125}I-STa to intact cells transfected with pNebo-STaR or with pNebo was measured in presence of increasing concentrations of unlabeled STa (Fig. 5). Specific competitive binding was observed for the STaR-expressing cells, whereas no binding over background was detected in pNebo-transfected cells. Scatchard analysis of the displacement data demonstrated the presence of a single high affinity binding site with a calculated K_d of approximately 1 nM (Fig. 5, *inset*). This value is comparable with the 1.4 nM K_d determined for ^{125}I-STa binding to rat brush border membranes (11).

DISCUSSION

The human STaR reported in this study has the same overall structural organization as the natriuretic peptide

[2] G. Cachianes and D. Leung, unpublished data.

FIG. 1. **Nucleotide sequence and deduced amino acid sequence of the human STaR.** Nucleotides are numbered at the beginning of each line. Numbers above the sequence refer to the amino acid sequence. The predicted cleavage site for the signal peptide is indicated by an *arrow*. Potentials *N*-linked glycosylation sites are *boxed*, and the predicted transmembrane domain is indicated by the *thick bar* over amino acids 411–431. Cysteine residues are indicated by a *solid dot* above the sequence.

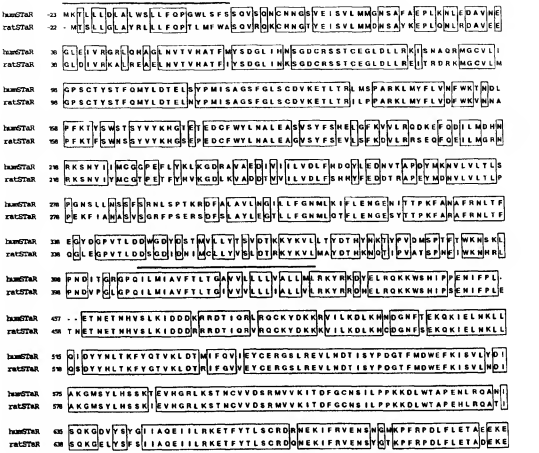

FIG. 2. **Amino acid sequence homology between the human and the rat STaR.** The predicted amino acid sequences of the human STaR is aligned with rat STaR (18). Gaps introduced for optimal alignment are shown by *dashes*. Identical amino acids are *boxed*. The signal sequence and the transmembrane domain are shown by *overlines*.

receptors ANPRA and ANPRB, adding a new member to the guanylyl cyclase receptor family. These receptors contain a single transmembrane domain that divides the molecule into an extracellular hormone binding region and an intracellular signalling domain. The intracellular domain contains a region of about 250 amino acids that has strong homology to the catalytic domain of many protein kinases. In the case of ANPRA, this domain has been shown to be required for ligand-dependent signaling (30). It is unclear, however, if this domain has intrinsic kinase activity.

Based on alignment of the amino acid sequences of STaR, ANPRA and ANPRB, the most conserved region is the guanylyl cyclase domain (~50% identity), followed by the kinase homology domain (~35% identity). The extracellular domain is less conserved with only 15 and 17% similarity with ANPRA and ANPRB, respectively. This low homology between the different extracellular domains suggests that the natural ligand for the STaR will probably not be a member of the natriuretic peptide hormone family. Moreover, none of the natriuretic peptides identified as yet (ANP, BNP, and CNP) is able to stimulate guanylyl cyclase activity in human 293 cells overexpressing the STaR. STa, on the other hand, stim-

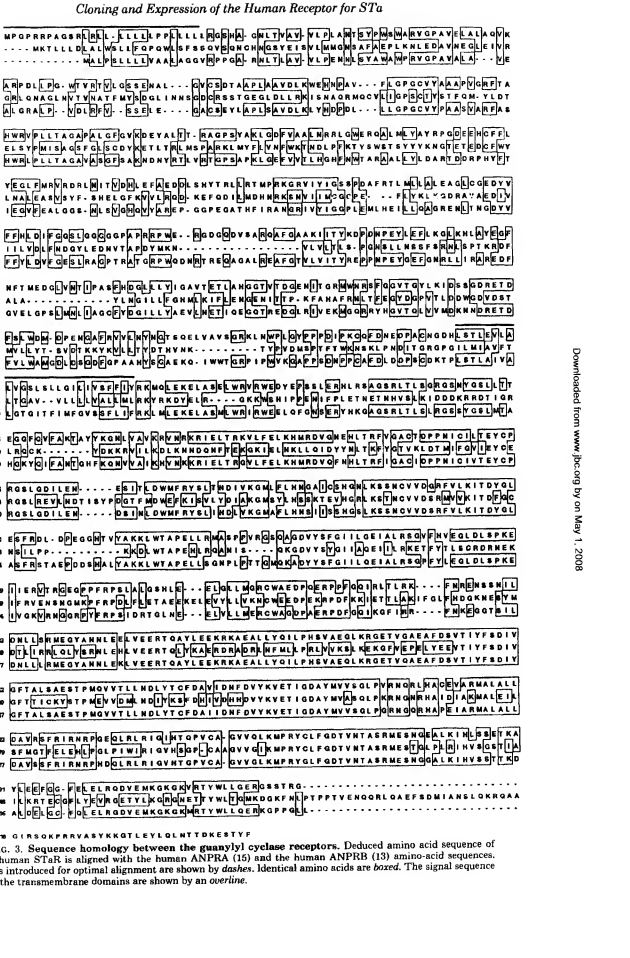

FIG. 3. Sequence homology between the guanylyl cyclase receptors. Deduced amino acid sequence of the human STaR is aligned with the human ANPRA (15) and the human ANPRB (13) amino-acid sequences. Gaps introduced for optimal alignment are shown by *dashes*. Identical amino acids are *boxed*. The signal sequence and the transmembrane domains are shown by an *overline*.

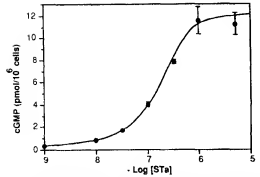

FIG. 4. **Dose-dependent stimulation of STaR overexpressing cells by STa.** Human 293 cells stably transfected with the pNeho-STaR expression plasmid were incubated with various concentrations of STa for 10 min. Intracellular cGMP accumulation was then determined. Each point represents the mean of triplicate samples assayed in duplicate and are represented ± S.E.

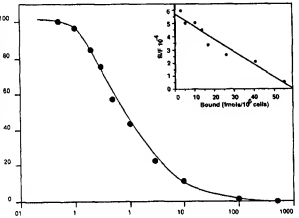

FIG. 5. **Inhibition of ^{125}I-STa binding to STaR-overexpressing cells.** Varying concentrations of unlabeled STa were incubated with 50 pM ^{125}I-STa and 5×10^5 G418-resistant 293 cells expressing STaR. Nonspecific binding was determined in the presence of 500 nM of STa. The percent specific binding is plotted *versus* the concentration of STa. Each point represents the mean of duplicate determination. A Scatchard analysis of the data is presented in the *inset*.

ulates cGMP accumulation in these cells with an EC_{50} of approximately 150 nM and binds with an affinity of ~1 nM.

The cDNA that we describe here encodes a receptor with both the STa binding site and the guanylyl cyclase catalytic site. These data are not consistent with previous results, indicating that the binding site and the catalytic site could be dissociated (11, 12). A large number of proteins have been identified in cross-linking experiments with STa (31), and our results do not rule out the possibility of heterogeneity among STa receptors. However, cross-linking experiments on opossum kidney cells with ^{125}I-STa reveal a single protein with an M_r of 120,000 (35), the predicted size for the STaR we report here.

Comparison of the predicted amino acid sequences of the human and the rat STaR sequences shows that the intracellular domain is more highly conserved (91% identity) than the extracellular domain (71% identity). The entire predicted amino acid sequences of ANPRA (92%) and ANPRB (98%) are highly conserved between rat and human, including all the glycosylation sites. This divergence of the STaR extracellular domain might be expected to reflect some species-specific interactions with the ligand but there appears to be little difference between the human receptor reported here and the rat receptor (12) interaction with a 19-amino acid STa peptide from *E. coli*. However, a putative endogenous ligand for these receptors may be species-specific.

The stable cell line expressing human STaR cDNA provides a useful tool to identify such a ligand or to design possible antagonists of the undesirable actions of STa. Antagonists that target the human receptor might be of therapeutic interest. To begin designing possible antagonists, information about the active portion of the STa peptide is valuable. In fact, it has been shown that only 13 amino acids out of the 19 or 20 are shown to be necessary for activity in suckling mice (32). This 13-amino acid sequence is very well conserved between the various bacteria that secrete heat-stable enterotoxins and is characterized by the presence of 6 cysteines, all of which are essential for activity (33). A large number of small peptides, known as conotoxins, have been purified from sea snail venoms (34) and found to have sequence similarity with the active portion of the heat-stable enterotoxins. However, one of the most homologous conotoxin, conotoxin GI (46% identity with the active portion of STa), does not bind to the STaR present on the rat intestinal epithelium (12).

STa stimulation of guanylyl cyclase was observed recently in various opossum epithelial cells other than intestine (35). This result suggests that an endogenous ligand for the STa receptor regulates guanylyl cyclase activity in organs that would not normally be exposed to STa. However, non-intestinal distribution of STaR has not been reported for other mammals. In the case of the rat, STaR mRNA was detected by northern blot analysis only in the intestine (19). Detailed information on the tissue-specific expression of this receptor await additional techniques such as *in situ* hybridization. Availability of cells lines overexpressing the STaR may help to identify the existence of a putative natural agonist for this receptor and to elucidate the role of this guanylyl cyclase receptor in intestinal cells.

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EXHIBIT 2

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Biochemistry

Precursor structure, expression, and tissue distribution of human guanylin

(enterotoxin/heat-stable enterotoxin receptor/Paneth cells/intestine)

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ABSTRACT **Heat-stable enterotoxins (STa) are small, cysteine-rich peptides secreted by *Escherichia coli* that are able to induce diarrhea through the stimulation of an intestine-specific receptor-guanylyl cyclase known as STaR. A 15-amino acid peptide, guanylin, was recently purified from rat jejunum and proposed to be a potential endogenous activator of this receptor. We describe here the cloning and characterization of human and mouse cDNAs encoding precursor proteins of 115 and 116 amino acids, respectively, having guanylin present at their C termini. Expression of the human cDNA in mammalian cells leads to the secretion of proguanylin, an inactive 94-amino acid protein. Guanylin generated by either trypsin or acid treatment of proguanylin was purified and found to bind to, and activate, STaR. Northern blot and *in situ* hybridization show high-level expression of guanylin mRNA restricted to the intestine, with localization to Paneth cells at the base of the small intestinal crypts. These results demonstrate that guanylin is an endogenous activator of STaR.**

The recent cloning and characterization of membrane-bound forms of guanylyl cyclases have demonstrated that they function as receptors for small peptides. These receptor-guanylyl cyclases share a common topological organization with a single transmembrane domain separating the extracellular binding domain from the intracellular region. The latter contains two distinct domains, one having homology to protein kinases followed by a C-terminal guanylyl cyclase catalytic domain. The first receptor-guanylyl cyclases to be identified were the natriuretic peptide receptors A and B (NPR-A and NPR-B) (1–4). Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) have been shown to selectively bind and stimulate these two homologous receptors (for review, see ref. 5). These natriuretic peptides, which share a similar structure with a conserved disulfide bond creating a 17-amino acid ring structure, are involved in the regulation of fluid and electrolyte homeostasis.

More recently, a third member of the receptor-guanylyl cyclase family that is specifically expressed in the intestine has been identified as the heat-stable enterotoxin receptor (STaR) (6, 7). Heat-stable enterotoxins (STa) are small peptides of 18 or 19 amino acids, secreted into the intestine by enterotoxigenic strains of *Escherichia coli* (8). The 13 amino acids necessary for the toxic activity of the peptide include 6 cysteines that form three disulfide bridges (9). Binding of STa to STaR induces a dramatic increase of the cGMP content of the cell (10, 11). This increase, in turn, inhibits salt absorption and stimulates chloride secretion. This imbalance of ions is accompanied by a massive accumulation of water in the gut that gives rise to the diarrhea and dehydration characteristic of enterotoxin activity (10).

The initial identification of a receptor for STa on intestinal brush border membranes (12) suggested the existence of an endogenous activator. Recently, guanylin, a 15-amino acid peptide purified from rat small intestine, was described as a potential ligand for the STaR (13). This peptide shares sequence similarity with STa, including four conserved cysteines. Furthermore, guanylin can compete with ^{125}I-labeled STa (^{125}I-STa) binding and stimulate cGMP production in T84 cells, a human colonic cell line known to express the STaR (14, 15). We report here the molecular cloning of the human and the mouse cDNAs encoding guanylin.¶ Their sequences reveal that guanylin is present at the C-terminal end of a larger precursor protein. Characterization of human proguanylin expressed in mammalian cells indicates that the 94-amino acid proguanylin is inactive. The biologically active guanylin can be released by either chemical or enzymatic treatment of proguanylin. We also show by Northern blot and *in situ* hybridization that the guanylin mRNA is specifically expressed in cells of the intestinal epithelium.

MATERIAL AND METHODS

cDNA Cloning. An oligonucleotide probe (5′-CCCAA-CACCTGTGA$\frac{G}{A}$ATCTG$\frac{C}{T}$GCCTATGCTGC$\frac{C}{T}$TGCACAG-GCTG-3′) was derived from the protein sequence for rat guanylin (13) using human codon bias (16) and was synthesized and labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. This probe was used to screen 10^6 clones of a human terminal ileum cDNA library in λgt10. Duplicate filters were hybridized under low stringency conditions at 42°C in 20% formamide, 5× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate), 10× Denhardt's, 0.05 M sodium phosphate (pH 6.5), 0.1% sodium pyrophosphate, 50 μg of sonicated salmon sperm DNA per ml, and 10% dextran sulfate. Filters were rinsed in 2× SSC and then washed once in 1× SSC/0.1% SDS at 42°C. Hybridizing phage were plaque-purified and the cDNA inserts were subcloned into the Bluescript plasmid (Stratagene). Both strands were sequenced by the dideoxy chain-termination procedure (17).

Expression. The full-length guanylin cDNA was subcloned into the mammalian cell expression vector pRK5 (R. Klein and D.V.G., unpublished data), under the control of the cytomegalovirus immediate early promoter. Human embryonic kidney 293 cells, maintained in Dulbecco's modified

Abbreviations: STa, heat-stable enterotoxin(s); NPR-A, natriuretic peptide receptor A; NPR-B, natriuretic peptide receptor B; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; STaR, STa receptor.

¶The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M95174 (human guanylin) and M95175 (mouse guanylin)].

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Eagle's medium (DMEM) supplemented with F-12 nutrient mixture, 20 mM Hepes (pH 7.4), and 10% fetal bovine serum, were transfected by the calcium phosphate method as described (18). Twenty-four hours following transfection the cells were rinsed with serum-free medium and then incubated for 4 hr in the presence of 200 μCi of [^{35}S]cysteine and [^{35}S]methionine per ml. Conditioned medium was collected and loaded on a Phast Hi-Density SDS/polyacrylamide gel (Pharmacia). After electrophoresis, the gel was dried and exposed to a storage phosphor imaging plate for 12 hr and then developed with a Fuji Bas-2000 bio-image analyzer (Fuji Medical Systems, Stamford, CT).

Purification of Guanylin. Fifty milliliters of serum-free medium conditioned for 48 hr was loaded on a Vydac C_{18} 300A column (4.7 × 250 mm) and eluted with a linear gradient of 10–40% acetonitrile in 0.1% trifluoroacetic acid (TFA) in 120 min. Fractions containing proguanylin were pooled and rechromatographed on a Synchrom C_4 4000A column (2 × 100 mm). Purified proguanylin was then digested with Promega modified trypsin using an enzyme-to-substrate ratio of 1:50 in 80 μl of 0.2 M NH_4HCO_3 (pH 8.0) for 1 hr at 37°C. The digest was directly injected on a Synchrom C_4 column and the peptides were separated by a linear gradient of 0.1% TFA to 70% acetonitrile/0.08% TFA in 20 min. The active fraction was rechromatographed on the same column using a linear gradient of 0.1% TFA/10% 1-propanol to 40% 1-propanol in 40 min. The sample was analyzed by electrospray mass spectroscopy, amino acid analysis, and protein sequencing.

Protein Sequencing, Amino Acid Analysis, and Mass Spectroscopy. Automated Edman degradation was performed on a 477 Applied Biosystems sequencer equipped with a 120A phenylthiohydantoin amino acid analyzer. Peaks were integrated with a Chrom Perfect (Justice Innovation, Palo Alto, CA) data system (19). Amino acid composition was determined after hydrolysis with 6 M HCl in a Millipore Pico-Taq workstation. The hydrolysates were dried and applied to a Beckman model 6300 amino acid analyzer equipped with ninhydrin detector. Electrospray spectra were obtained on a Sciex APIII triple quadrapole mass spectrometer. Spectra were obtained by direct infusion at a flow rate of 1.5 μl/min.

Binding and cGMP Assay. Competition of ^{125}I-STa binding and guanylyl cyclase stimulation of 293-STaR cells were measured as described (7, 15).

Northern Blot. Approximately 20 μg of total RNA (intestine) or 2 μg of poly(A)$^+$ RNA from various human tissues (Stratagene) was electrophoresed into a formaldehyde/1.2% agarose gel and blotted onto a nylon membrane. The membrane was hybridized with a ^{32}P-labeled guanylin cDNA fragment. The blot was washed at 65°C in 0.1× SSC/0.1% SDS and then exposed overnight to a storage phosphor imaging plate.

***In Situ* Hybridization.** *In situ* hybridization was performed essentially as described (20). ^{35}S-labeled riboprobes were generated from appropriately linearized plasmids containing a full-length cDNA clone of human guanylin. Hybridization was carried out overnight at 55°C in a hybridization buffer containing 50% formamide, 0.3 M NaCl, 1× Denhardt's, 25 μg of tRNA per ml, 10 mM dithiothreitol, and probe at 5 × 10^5 cpm/μl. After hybridization, slides were washed as follows: 50% formamide, 2× SSC, 1× Denhardt's at 55°C, two washes in 2× SSC at room temperature, RNase A at 37°C in 2× SSC, 2× SSC at 42°C, and finally 0.1× SSC at 55°C. Washed, dehydrated slides were dipped in Kodak NBT emulsion and exposed at 4°C for 6–12 days before developing and counterstaining with hematoxylin/eosin.

RESULTS

cDNA Cloning. A DNA probe was synthesized based on the amino acid sequence of rat guanylin (13) using human codon bias and was used to screen a human terminal ileum cDNA library. Several positive clones were identified and five were found to contain inserts of about 600 base pairs (bp). DNA sequencing indicated that these five clones differed only by the length of their poly(A) tails and 5′ untranslated regions. The presumed initiation codon at position 5–7 in the longest clone (Fig. 1) is within a consensus sequence favorable for eukaryotic translation initiation (21) and defines the beginning of an open reading frame of 115 amino acids. The N terminus of the predicted amino acid sequence is highly hydrophobic and probably corresponds to a signal peptide. The consensus rules described by von Heijne (22) indicate potential cleavage sites at positions 16 and 21, generating prohormones of 99 amino acids (10,712 Da) or 94 amino acids (10,343 Da). The prohormone contains six cysteines, an unusually high number of prolines, 12, as well as one potential N-linked glycosylation site. The C terminus of the protein corresponds to the published rat guanylin sequence except for the glycine at position 102 which is asparagine in the rat sequence. The TAG stop codon is followed by 215 nucleotides of 3′ untranslated sequence. A poly(A) consensus signal AAUAAA (23) is found at position 548. In a homology screen, neither the cDNA nor the predicted amino acid sequence showed any significant homology with sequences present in the Genbank or Dayhoff data bases.

A cDNA fragment corresponding to the coding region of human guanylin was used to screen a mouse small intestine cDNA library of a million clones. Approximately 200 positive plaques were identified and 2 of them were sequenced.

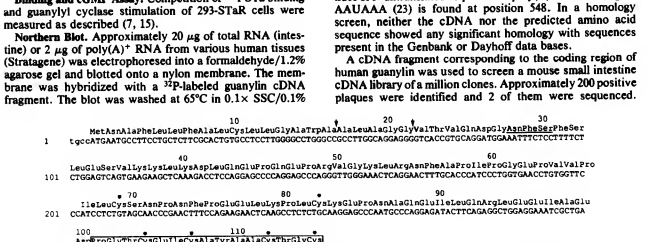

FIG. 1. Nucleotide sequence and deduced amino acid sequence of human guanylin. Nucleotides are numbered at the beginning of each line. Numbers above the sequence refer to the amino acid sequence. The predicted signal peptide cleavage sites are indicated by arrows. The potential N-linked glycosylation site is underlined and cysteine residues are indicated by a solid dot above the sequence. The amino acid sequence corresponding to the purified rat guanylin (13) is boxed.

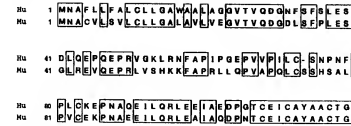

FIG. 2. The predicted amino acid sequences of human (Hu) and mouse (Mu) guanylin are aligned. A gap introduced into the human sequence for optimal alignment is shown by a dash. Identical amino acids are boxed.

Alignment of the human and the mouse amino acid sequences (Fig. 2) indicates that their C-terminal sequences are highly conserved (87% homology between amino acids 85 and 115). The rest of the molecule is much less conserved, with only 58% identity between the first 84 residues. Both cysteines in the precursor region are conserved as well as 8 of the 11 prolines present in the human prohormone. An 8-amino acid motif (LESVKKLK) is present in both sequences at position 33.

Expression. The full-length human cDNA was inserted in the expression vector pRK5 and transfected into the human embryonic kidney 293 cell line. Twenty-four hours later conditioned medium was analyzed for the presence of novel proteins by metabolic labeling and SDS/PAGE (Fig. 3). A 10-kDa protein was detected in the supernatant of cells transfected with pRK-guanylin but not in those transfected with pRK alone. However, the proguanylin-containing conditioned medium was not able to inhibit the binding of ^{125}I-STa binding to 293-STaR cells.

To further characterize the properties of guanylin, human proguanylin was purified from 50 ml of conditioned medium by reverse-phase HPLC. N-terminal sequence analysis and electrospray mass spectrometric analysis (10,337 Da) indicate that the prohormone sequence starts at residue 22, the second predicted cleavage site for the signal peptide, that it is not glycosylated, and that all six cysteines are involved in disulfide bridges. The proguanylin was digested with trypsin and the peptides generated were separated on a C_4 reverse-phase column. The fraction containing the 22-amino acid (2258 Da) C-terminal fragment was characterized by mass spectrometry. The electrospray mass spectrum contains a single ion signal at m/z 1130 that corresponds to the diprotonated peptide-ion. Amino acid sequencing revealed the presence of a single peptide with the correct sequence. Amino acid analysis indicated a yield of ≈12 nmol/50 ml of conditioned medium.

The ability of the 22-amino acid peptide to compete with ^{125}I-STa for binding to STaR was studied by incubating intact 293-STaR cells with 25 pM ^{125}I-STa and increasing concentrations of guanylin (Fig. 4*A*). Fifty percent displacement of bound ^{125}I-STa was observed with 100 nM guanylin. To measure the capacity of guanylin to stimulate guanylyl cyclase activity, 293-STaR cells were treated with increasing concentrations of guanylin and the accumulation of intracellular cGMP was measured. In unstimulated cells, 0.6 pmol of cGMP per 10^6 cells was detected. This concentration increased 75-fold to 45 pmol per 10^6 cells in the presence of 0.01 mM guanylin (Fig. 4*B*). With STa, maximal stimulation of 293-STaR cells was obtained at 0.1 μM, where the cGMP

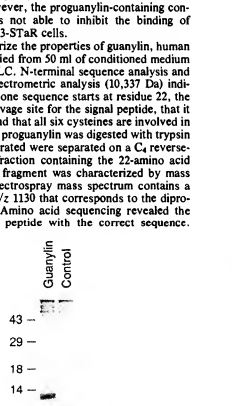

FIG. 3. SDS/PAGE analysis of ^{35}S-labeled proteins from the supernatant of 293 cells that were transiently transfected with pRK or with pRK-guanylin. Molecular mass markers (kDa) are indicated.

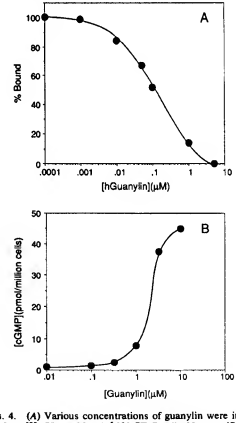

FIG. 4. (*A*) Various concentrations of guanylin were incubated with 25 pM ^{125}I-STa and 2×10^5 293-STaR cells. Nonspecific binding was determined in the presence of 5 μM guanylin. The percent specific binding is plotted versus the concentration of STa. Each point represents the mean of duplicate determinations. (*B*) 293-STaR cells were incubated with various concentrations of guanylin for 30 min. Intracellular cGMP accumulation was then determined. Each point represents the mean of duplicate samples assayed in duplicate.

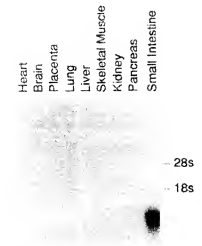

FIG. 5. Northern blot analysis of human guanylin steady-state mRNA levels. RNAs prepared from different human tissues were fractionated on a 1.2% denaturing agarose gel and blotted to nylon. The blot was probed with a 270-bp cDNA fragment corresponding to the coding region of human guanylin.

concentration increased 153-fold to 92 pmol per 10^6 cells (data not shown).

Tissue Distribution of Guanylin. To study the tissue distribution of guanylin, we analyzed RNA from various adult human tissues by Northern blot. A single abundant guanylin transcript of ≈0.7 kilobase was detected only in the small intestine (Fig. 5). Cell-specific expression of guanylin was studied in the small intestine by *in situ* hybridization to sections of human tissue. Strong hybridization of the guanylin antisense probe to cells at the base of the intestinal crypts was observed (Fig. 6*A*). Cells hybridizing to the guanylin probe contained prominent eosinophilic granules in their apical cytoplasm and were located in the same position as cells in parallel sections that hybridized to a human lysozyme probe (data not shown). This indicates that the guanylin-positive cells in the small intestine are Paneth cells (20). Sections hybridized to sense strand riboprobes showed no hybridization signal (Fig. 6*B*).

DISCUSSION

The receptor-guanylyl cyclases are a family of proteins that can be activated by small peptide ligands. The most recent member of the family to be identified is STaR (6, 7), an intestinal receptor for the STa secreted by enterotoxigenic strains of *E. coli*. However, no endogenous ligand able to regulate this receptor had been identified. Recently, a 15-amino acid peptide called guanylin was purified from rat intestine and shown to bind and stimulate the STaR present on T84 cells (13). We report here the cloning of the human and the mouse cDNAs encoding this hormone, confirming that guanylin is of intestinal origin and was not purified from a bacterial contamination of the intestinal extracts. The cDNA sequences revealed that guanylin is the C-terminal end of a larger proguanylin molecule. The natriuretic peptides ANP, BNP, and CNP, which activate the NPR-A and NPR-B receptor-guanylyl cyclases, are also the C-terminal portions of longer precursors. Interestingly, the hormone-containing C-terminal region is highly conserved between human and mouse proguanylin, whereas the N-terminal part of the precursor is much less conserved.

Transfection of the full-length cDNA in human 293 cells leads to the secretion of an inactive 10-kDa protein, indicating that these cells are unable to correctly process the prohormone. The proline previously reported (13) as the first amino acid of the purified guanylin probably does not correspond to the N terminus of the natural hormone. This active peptide was probably generated by cleavage at the Asp-100–Pro-101 bond during the acetic acid boiling step used in the purification process. The only basic residue present in the highly conserved region between the human and the mouse sequences, Arg-93, is more likely to be a physiologic processing site. Therefore we chose to digest proguanylin *in vitro* with trypsin. Cleavage at Arg-93 generates a 22-amino acid C-terminal peptide that is a competitive inhibitor of ^{125}I-STa binding and stimulates cGMP production in 293-STaR cells. However, as for the previously purified 15-amino acid peptide, relatively high concentrations are required for half-maximal displacement of ^{125}I-STa binding (0.1 μM) and

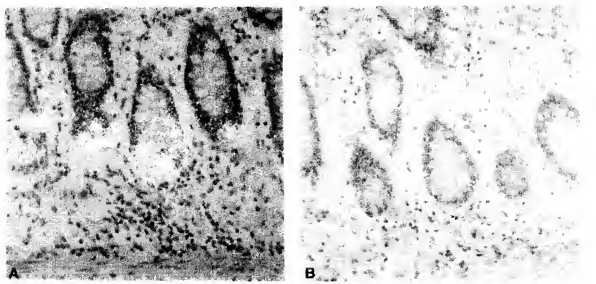

FIG. 6. *In situ* hybridization to human small intestine. ^{35}S-labeled human guanylin antisense (*A*) and sense (*B*) strand riboprobes were hybridized to sections of human small intestine. Sections were exposed for 7 days, counterstained with hematoxylin/eosin, and viewed by epiluminescence and bright-field microscopy. (×60.)

half-maximal cGMP stimulation (2 μM). Even the high levels of guanylin mRNA expression detected in the intestine by Northern blot are unlikely to result in the synthesis of micromolar concentrations of guanylin. Therefore, it may not be possible for the receptor to be maximally stimulated by guanylin *in vivo*. The large amounts of cGMP produced at 0.01 mM guanylin are comparable to those produced at 0.1 μM STa (45 pmol per 10^6 cells versus 92 pmol per 10^6 cells) and might result in diarrhea such as is caused by STa. It is also possible that, *in vivo*, proguanylin is processed at a different site, generating a ligand with greater potency. Dibasic sites are among the most common processing sites in mammals and are cleaved by specific proprotein converting enzymes (24). The Lys-37–Lys-38 dipeptide, which is found within an 8-amino acid motif that is identical in the mouse and human sequences, could represent such a site. However, the use of processing sites upstream from the Arg-93 would generate hormone sequences that would be less well conserved between human and mice and that might exhibit species specificity. Alternatively, there may be another, as yet unidentified, ligand with a higher affinity for STaR. For example, NPR-A has two ligands, ANP and BNP, that bind to the receptor and stimulate cGMP production, but ANP is 10-fold more potent than BNP (4).

Northern blot analysis of human mRNA did not reveal expression in tissues other than the intestine. However, more extensive *in situ* hybridization analysis or the use of more sensitive techniques such as PCR may demonstrate expression in other tissues, as a small amount of guanylin-like activity was detected in the rat kidney (13). Alternatively, study of other tissues may lead to the identification of guanylin-related ligands that signal through the STaR or other related receptor-guanylyl cyclases.

As evidenced by the severe secretory diarrhea resulting from infection with enterotoxin-expressing bacteria, activation of intestinal guanylyl cyclase can have profound effects on fluid balance in the intestine. Guanylin and perhaps related molecules are likely to be the endogenous mediators regulating secretion in the small and large intestine under physiological and pathological conditions. In the latter case, increased intestinal mobility and copious secretion may constitute a defense mechanism against potentially invasive microorganisms. This interpretation is consistent with the expression of guanylin in Paneth cells of the small intestine. As Paneth cell numbers are increased in response to inflammation and bacterial colonization, it is thought they play a significant role in regulating the fauna of the small intestine. Furthermore, Paneth cells synthesize a small group of proteins (lysozyme, cryptdin, defensins) that are primarily microbicidal (25, 26). However, Paneth cells also express other genes not directly involved in microbial killing, such as tumor necrosis factor (20) and epidermal growth factor (27). Thus guanylin may have other effects on epithelial cells that remain to be defined. It will be of great interest to examine the expression of guanylin in human disease and in animal models of intestinal pathology.

We thank M. Struble for protein purification, J. Bourell for mass spectroscopy analysis, M. Vasser, P. Jhurani, P. Ng, and F. Hsu for oligonucleotide synthesis, C. Chan for *in situ* hybridization, D. Drayna and D. Lowe for RNA samples, V. Gibbs for tissue specimens, Q. Gu for DNA sequencing, and R. Horuk, D. Marriott, and C. Garcia for helpful discussions. S.K. is the Staines Medical Research Fellow at Exeter College, Oxford, and was supported by a Medical Research Council project grant.

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EXHIBIT 3

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Guanylyl Cyclase C Is an *N*-Linked Glycoprotein Receptor That Accounts for Multiple Heat-stable Enterotoxin-binding Proteins in the Intestine*

(Received for publication, April 28, 1992)

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Guanylyl cyclase C (GC-C) is a newly discovered receptor found in the intestine, and possibly in other epithelia, that binds bacterial heat-stable enterotoxins (STa). The receptor has now been stably expressed in human embryonic 293 cells, which do not normally contain the receptor. Cyclic GMP concentrations are elevated 40-fold in response to 1 μM STa, and membranes obtained from the overproducing cells contain GC-C activity that can be stimulated about 9-fold by STa alone and an additional 1.4- to 2-fold by a combination of ATP and STa. The ATP effect does not appear to be due to enzyme activation, but instead to protection of GC-C against inactivation. Antibody raised against the carboxyl-terminal sequence of GC-C identified two major proteins (M_r 140,000 and 160,000) in 293 cells expressing GC-C. Both proteins bound to wheat germ lectin-Sepharose, and *N*-glycosidase F treatment converted both forms to a single M_r 120,000 protein, the size predicted from amino acid composition. The addition of high concentrations of tunicamycin to 293-GC-C cells also reduced the M_r to 120,000, indicating that GC-C is an *N*-linked glycoprotein. When rat intestinal membranes or 293-GC-C cells were cross-linked with ^{125}I-labeled STa, the major ^{125}I-labeled protein complexes had M_r ranging between 45,000 and 80,000. On immunoblots of rat intestinal membranes treated with a reducing agent, 3 major proteins of M_r 65,000, 85,000, and 140,000 were specifically recognized by a GC-C antibody. However, under nonreducing conditions one major GC-C related protein appeared at a higher position (M_r 230,000). Its mobility was reduced in the presence of STa, similar to rGC-C expressed in 293 cells. These data indicate that at least part of the lower M_r STa-binding proteins found in intestinal extracts represent proteolytic products of GC-C.

Secretory diarrhea caused by pathogenic strains of *Escherichia coli* is a major cause of infant deaths in developing countries (1). The enterotoxins secreted by these bacteria activate signal transduction pathways normally exploited by endogenous neurohormonal regulators of intestinal salt and water transport (2). *E. coli* heat-stable enterotoxins (STa)[1] are low molecular weight peptides known to activate intestinal guanylyl cyclase (GC) after binding to specific surface receptors (2, 3); they may mimic the action of a recently discovered endogenous peptide, guanylin, extracted from rat jejunum (4). The guanylin sequence exists within an 0.8–0.9-kilobase mRNA that is present not only in small intestine, but also in the kidney, adrenal gland, and uterus/oviduct of the rat (5). The rise in cGMP subsequently leads to a stimulation of net fluid secretion through the activation of Cl^- channels and the inhibition of coupled NaCl transporters (1–3). A unique type II isoform of cGMP-dependent protein kinase, present in the apical membrane of the enterocyte, likely plays a role in the cGMP provoked secretion (6). Previously, the form of guanylyl cyclase activated by STa was thought to be intestine-specific, cytoskeleton-associated, and distinct from STa receptors (7, 8). However, molecular cloning of guanylyl cyclases from rat and human intestinal cDNA libraries, and subsequent expression of these clones in mammalian cells, revealed that an intestinal guanylyl cyclase (GC-C), in fact, was a STa receptor (9–11). However, the nature of the lower M_r STa-binding proteins, whose M_r range between 55,000 and 80,000, has remained unclear (8, 12, 13). Whether they represent a novel class of STa receptors, or proteins related to GC-C, has not been established. In the present study, GC-C was characterized in overexpressing fetal human kidney 293 cells and in intestinal membranes by means of a monospecific antibody, and by chemical cross-linking with ^{125}I-STa. The results obtained with both methods suggest that some if not all of the lower M_r STa-binding proteins found in intestinal extracts are proteolytic products of GC-C. Our studies also show that GC-C is an *N*-linked glycoprotein, and that in contrast to the natriuretic peptide receptors (14–16), ATP is not required for STa receptor-cyclase interaction.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and G418 sulfate (Geneticin®) were obtained from Gibco, *E. coli* heat-stable enterotoxin (STa), 3-isobutyl-1-methylxanthine (IBMX), and wheat germ agglutinin (WGA)-Sepharose were from Sigma, *N*-glycosidase F from Boehringer Mannheim, and $Na^{125}I$, and L-[^{35}S]methionine (EXPRE^{35}S^{35}S, protein labeling mixture) from Du Pont-New England Nuclear. Protein standards (myosine, M_r 200,000; β-galactidase, M_r 116,000; phospho-

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[1] The abbreviations used are: STa, *E. coli* heat-stable enterotoxin; GC-C, guanylyl cyclase C; PBS, phosphate-buffered saline; WGA, wheat germ agglutinin; IBMX, 3-isobutyl-1-methylxanthine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium.

rylase *b*, *M*, 97,000; albumin: bovine, *M*, 67,000; egg, *M*, 45,000) were from Bio-Rad, and thyroglobulin, subunit *M*, 330,000, from Pharmacia LKB Biotechnology Inc. Tunicamycin, deoxymannojirimycin, and swainsonine were a gift from Dr. M. Lehrman.

Stable Transfection of 293-cells with rGC-C—Full-length cDNA coding for rGC-C (8) was cloned into the *Hin*dIII and *Not*I sites of the expression vector pRc/CMV (Invitrogen, San Diego, CA) which contains a CMV promoter and a neomycin selectable marker. Human embryonic kidney 293 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. The cells were transfected at 50% confluency (10 μg of DNA/100-mm plate) with Lipofectin™ (Bethesda Research Laboratories Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. After selection with G418 sulfate (400 μg/ml) for 10 days, 40 surviving clones were separately subcultured and tested for STa stimulation of cGMP levels.

Preparation of 293-rGC-C and Intestinal Brush Border Membranes—Confluent 293-rGC-C cells grown on 100-mm dishes were washed twice with 10 ml of ice-cold phosphate-buffered saline (PBS) and removed with a rubber policeman. The cells from 1 plate were resuspended in 1 ml of buffer A (100 mM NaCl, 50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 10% glycerol, 10 μg/ml pepstatin, and 20 μg/ml leupeptin) and homogenized by brief sonication (3 × 3 s, tip amplitude 15–20 μm). Membranes were pelleted at 20,000 × *g* for 15 min and washed twice with 1 ml of buffer A. The 293-rGC-C membranes were finally resuspended in 1 ml of buffer A without glycerol or EDTA at a concentration of 0.5–1.0 mg of protein/ml.

Jejunum and ileum were removed from rats under light ether anaesthesia and rinsed with ice-cold saline. The epithelial cells were scraped from the mucosal surface of the intestine with a glass slide, suspended in 100 ml of buffer containing 5 mM EDTA/imidazole, and washed 3 times with the same buffer. The cells from 1 intestine were finally resuspended in 20 ml of 300 mM mannitol, 10 mM Tris/HCl, pH 7.4, 10 μg/ml pepstatin, and 20 μg/ml leupeptin, and frozen in liquid N_2. After thawing, a brush border membrane-enriched fraction was obtained by differential $MgSO_4$ (10 mM) precipitation (two times) as previously described (17). Brush border membranes form tightly closed right-side out vesicles (17). Open brush border caps were prepared by vibration of everted rat small intestine in hypotonic EDTA buffer (18).

^{125}I-STa Binding and Cross-linking—STa was radioiodinated to specific activities of approximately 1300 Ci/mmol using Enzymobeads (Bio-Rad) (19), and binding experiments were performed on 293-rGC-C cells grown in 12-well tissue culture plates (Costar) (9). For cross-linking, membranes (200 μg of protein) were incubated for 30 min at 37 °C in 500 μl of DMEM, pH 6.0, containing 0.1% bovine serum albumin and 0.5 μCi of ^{125}I-STa in the presence or absence of 1 μM unlabeled STa. After centrifugation (15 min, 100,000 × *g*) pellets were resuspended 100 μl of DMEM, pH 7.4, and 1 mM disuccinimidyl suberate in dimethyl sulfoxide was added from a 100 mM stock. After incubation for 30 min at 37 °C, membranes were washed, resuspended in 50 μl of SDS sample buffer, and analyzed by SDS-PAGE followed by autoradiography.

Guanylyl Cyclase Assay and Cyclic GMP Determinations—Membranes (25–50 μg of protein) were incubated in a final volume of 0.1 ml containing 100 mM NaCl, 50 mM Tris/HCl pH 7.4, 0.25 mM IBMX, 10 mM $MgCl_2$, 5 mM creatine phosphate, 3–5 units of creatine phosphokinase, and 1 mM GTP at 37 °C. For determination of GC activity in brush border membranes and intestinal cells, 0.5% Triton X-100 was added to permeabilize the membranes/cells and 2 mM ATP to stabilize GC in the presence of the detergent. The reaction was stopped by the addition of 1 ml of 0.1 M HCl. After centrifugation (10 min, 20,000 × *g*) the supernatant fluid was neutralized with an equal volume of 0.09 M Tris, 2 mM EDTA. The samples were acetylated and cGMP was determined by radioimmunoassay (20).

For determination of cGMP levels, 293-rGC-C cells grown on culture plates were washed with a modified Meyler buffer (108 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20 mM $NaHCO_3$, 0.8 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 20 mM Na-HEPES, 10 mM glucose, pH 7.4) and incubated for 10 min at 37 °C in the same buffer containing 0.5 mM IBMX. The medium was aspirated and the cells were incubated for another 10 min with the same buffer containing IBMX and the compounds to be tested. The reaction was stopped by aspiration of the buffer and lysis of the cells in 0.1 M HCl. The cGMP was determined in the HCl-containing fraction after centrifugation and neutralization as described above. Levels or rate of formation of cGMP are expressed as means ± S.E. (n = number of individual experiments).

Immunoprecipitations and Immunoblots—A synthetic peptide, NNSDHDSTYF, representing the carboxyl-terminal sequence of rGC-C was synthesized with *tert*-butoxycarbonyl amino acids and coupled to the purified protein derivative of tuberculin (Statens Serum Institut, Copenhagen, Denmark) using 0.042% glutaraldehyde in PBS. Rabbits were immunized subcutaneously with the antigen (21). The antiserum with the highest titer against the peptide in enzyme-linked immunosorbent assay, Z659, was subsequentially used in this study.

For immunoprecipitation, cells grown on 30-mm culture plates were incubated at 37 °C for 4 h in 0.5 ml of DMEM without methionine, supplemented with 2% dialyzed fetal calf serum and 0.25 mCi/ml L-[^{35}S]methionine (1200 Ci/mmol). Cells were washed twice with 1 ml of ice-cold PBS and solubilized in 0.5 ml of buffer containing 100 mM NaCl, 50 mM Tris/HCl, pH 7.4, 10% glycerol, 10 μg/ml leupeptin, and 1% Triton X-100. After centrifugation (20 min, 100,000 × *g*), 400 μl of the supernatant fluid was incubated for 1 h with 2 μl of preimmune serum (final 1:200) and subsequently with 25 μl of protein A-Sepharose (Pierce Chemical Co.) for 1 h. After a short centrifugation, the supernatant fluid was split, and 200 μl were incubated for 1 h with 1 μl of Z659 antiserum (final 1:200) or with the same antiserum previously incubated for 1 h with the antigenic peptide (0.5 mg/ml). The proteins which bound to antibody were precipitated with 12.5 μl of protein A-Sepharose (1 h) and washed 4 times with 0.5 ml of solubilization buffer containing 1% sodium cholate and 0.1% SDS. They were finally resuspended in 50 μl of sample buffer plus 70 mM β-mercaptoethanol and boiled for 3 min prior to SDS-PAGE (22). The precipitated proteins (20 μl) were separated on 7.5% gels, and visualized after treatment of the gel with Enhance (Du Pont-New England Nuclear) by autoradiography using Hyperfilm (Amersham Corp.).

For the immunoblots, samples (approximately 20 μg of protein) were either resuspended in sample buffer with 70 mM β-mercaptoethanol and boiled for 3 min (reducing conditions), or resuspended in sample buffer without β-mercaptoethanol and incubated for 3 min at 65 °C (nonreducing conditions). The samples were separated by SDS-PAGE, and transferred to nitrocellulose (Sartorius). The immunoreactive proteins were detected after incubation for 1 h with antiserum Z659 (1:300) by the enhanced chemiluminescence method as described by the manufacturer (Amersham). In some experiments the antiserum was eluted from specific bands on the immunoblots by two subsequent 30-s incubations with 0.5 ml of 5 mM glycine/HCl, pH 2.3, 0.5% Tween 20, 0.5 M NaCl, and 0.01% bovine serum albumin. The eluate was subsequently neutralized with 20 μl of 1 M sodium phosphate buffer, pH 6.0.

RESULTS AND DISCUSSION

To investigate the phenotype of the cloned rat STa receptor/guanylyl cyclase (rGC-C), we stably expressed the protein in the human embryonic kidney 293 cells. The parental 293 cells have no apparent STa-binding sites and their intracellular cGMP levels are not detectably increased by STa (10) or natriuretic peptides (not shown). After transfection with the full-length rGC-C clone in an expression vector containing a cytomegalovirus promoter and a neomycin selectable marker, G418 sulfate-resistant clones were selected, and the clone with the highest STa-stimulated cGMP elevation was used for subsequent studies. This clone, referred to as 293-rGC-C, contained ^{125}I-STa-binding sites, and the binding could be inhibited in the presence of unlabeled STa with a half-maximal inhibition of 3.5 ± 1.5 nM, which is similar to that seen in COS cells transiently transfected with rGC-C (9) or in rat intestinal membranes (8).

STa (1 μM) caused an increase in the cGMP level of 293-rGC-C cells from 1.5 ± 0.3 to 68 ± 25 pmol/mg protein (n = 3) during a 10-min incubation in the presence of 0.5 mM IBMX. The guanylyl cyclase (GC) of isolated 293-rGC-C membranes could be stimulated approximately 9-fold by STa, the cGMP produced being 31 ± 8 pmol of cGMP/10 min/mg of protein under basal conditions and 295 ± 43 pmol of cGMP/10 min/mg of protein (n = 4) in the presence of 1 μM STa.

The stimulation of GC activity in 293-rGC-C membranes by STa is comparable to the stimulation (5–7-fold) observed in rat intestinal membranes (18, 23).

Another guanylyl cyclase receptor, GC-A, requires adenine nucleotides for full activation by its ligand, atrial natriuretic peptide (16). As shown in Fig. 1, ATP (1 mM) enhanced the STa-stimulated GC activity in 293-rGC-C membranes an additional 1.4–2-fold. However, unlike the data obtained with GC-A, the extended linear time course of GC-C stimulation by STa in the presence of ATP suggests that the ATP stimulation may be largely caused by a protection of GC-C against inactivation. This suggestion is corroborated by the finding that incubation of 293-rGC-C membranes for 6 min at 37 °C with STa (1 μM) lowers subsequent STa-stimulated GC activity by 85% (Fig. 2). A similar incubation in the absence of STa causes a smaller inhibition of the subsequent STa-stimulated GC activity, indicating that STa promotes the inactivation of GC-C at 37 °C (Table I). ATP largely protects against this inactivation when added together with STa (see Fig. 2, Table I), but not when added after the STa incubation and during the enzyme assay (Table I). This indicates that the protective effect of ATP is not caused by prevention of GTP degradation during the assay. The effects of ATP could be due to protection against STa-induced denaturation or desensitization, or to an acceleration of sensitization. Since the initial rate of cGMP formation is similar in the presence or absence of ATP (see Fig. 1), and since the STa-induced decrease in GC-C activity is not reversed by subsequent addition of ATP (Table I), the effects are likely due to ATP protection against STa-induced inactivation. The protective effect of ATP was half-maximal at 0.25 ± 0.1 mM ($n = 3$). Therefore, concentrations of ATP within the physiological range may be required to allow the persistence of a STa-provoked rise in intracellular cGMP in intact cells. The relatively large stimulation of rGC-C by STa alone, and the relatively small effect of ATP on this stimulation at the early time points, may suggest that adenine nucleotides do not play a direct role in 293 cell rGC-C activation. In this respect GC-C seems to differ from the natriuretic peptide receptor/GC-A, whose activation is regulated by ATP (15, 16). A glycine-rich loop which has been suggested to anchor the γ-phosphate of ATP in protein kinases (24), is present in the protein kinase-like domain of GC-A, and has been suggested to play a role in the regulation of GC-A activity by ATP (25); this region is absent in GC-C (9). An apparent STa-induced inactivation of GC-C, and the protective effect of ATP, has been observed also in isolated brush border caps from rat intestine (not shown), demonstrating that 293-rGC-C cells express a functional STa receptor with properties similar to those of intestinal membranes.

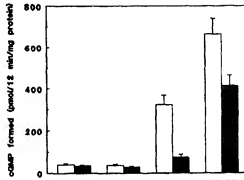

FIG. 2. **ATP protects GC-C from STa-induced inactivation.** Membranes from 293-rGC-C cells were incubated for 12 min at 37 °C as described under "Experimental Procedures" in the presence of 1 mM GTP (−), or in the presence of 1 mM ATP, 1 μM STa, or 1 mM ATP + 1 μM STa. Membranes were either added to the reaction mixture containing GTP (*open bars*), or incubated in the absence of GTP for 6 min at 37 °C in a reaction mixture containing STa, ATP, or ATP + STa. GTP was then added at 6 min to each mixture from a concentrated stock (*hatched bars*). Data are expressed as means ± S.E. of three experiments.

TABLE I

STa-induced inactivation of GC-C is not reversed by addition of ATP

Membranes from 293-rGC-C cells were incubated for 10 min at 37 °C as described under "Experimental Procedures" in the presence of 1 mM GTP plus the reagents mentioned under the incubation conditions (after GTP addition). Membranes were either added to the reaction mixture containing GTP (no incubation), or incubated in the absence of GTP for 6 min at 37 °C in the reaction mixture containing the extra additions mentioned in the table. GTP and the various other reagents were then added at 6 min to each mixture from a concentrated stock. Data are expressed as means ± S.E. of three experiments.

Incubation conditions		GC activity
Before GTP addition	After GTP addition	
6 min	*10 min*	*pmol cGMP formed/ mg protein/ 10 min*
No incubation	1 μM STa	313 ± 82
No incubation	1 mM ATP + 1 μM STa	617 ± 141
No addition	1 μM STa	217 ± 48
1 μM STa	1 μM STa	90 ± 22
1 μM STa	1 mM ATP + 1 μM STa	119 ± 24
1 mM ATP	1 mM ATP + 1 μM STa	583 ± 151
1 μM STa + 1 mM ATP	1 mM ATP + 1 μM STa	502 ± 118

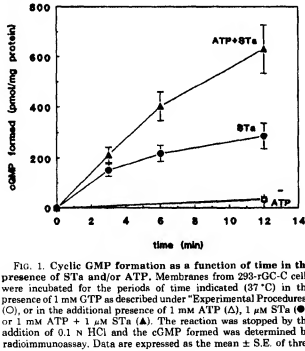

FIG. 1. **Cyclic GMP formation as a function of time in the presence of STa and/or ATP.** Membranes from 293-rGC-C cells were incubated for the periods of time indicated (37 °C) in the presence of 1 mM GTP as described under "Experimental Procedures" (○), or in the additional presence of 1 mM ATP (△), 1 μM STa (●), or 1 mM ATP + 1 μM STa (▲). The reaction was stopped by the addition of 0.1 N HCl and the cGMP formed was determined by radioimmunoassay. Data are expressed as the mean ± S.E. of three experiments.

The recombinant rGC-C protein expressed in 293 cells was further characterized by immunoprecipitation with an antiserum raised against a synthetic decapeptide, representing the carboxyl-terminal sequence of rGC-C. As shown in Fig. 3, this antiserum (Z659) specifically precipitated 140- and 160-kDa

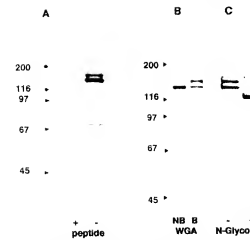

FIG. 3. **Characterization of rGC-C by immunoprecipitation.** *Panel A*, 293-rGC-C cells were labeled with [^{35}S]methionine then subsequently solubilized in 1% Triton X-100. The proteins were then precipitated with anti-GC-C serum (−) or with antiserum preabsorbed with the GC-C specific peptide (+). *Panel B*, solubilized proteins from [^{35}S]methionine-labeled 293-rGC-C cells were incubated (250 μl) with wheat germ agglutinin-Sepharose (1 mg of WGA/ml) for 1 h. The WGA-Sepharose was then washed 3 times with solubilization buffer by centrifugation, and then incubated in the same buffer containing 0.2 M *N*-acetylglucosamine (30 min). After a final centrifugation, the supernatant fluids, containing, respectively, non-WGA-bound (*NB*) and specific WGA bound proteins (*B*), were immunoprecipitated with antiserum to rGC-C. *Panel C*, immunoprecipitated rGC-C bound to protein A was incubated for 10 min at 95 °C in 40 μl of PBS containing 0.5% SDS and 1% β-mercaptoethanol. After a short centrifugation, the supernatant fluid containing rGC-C was diluted 4-fold with PBS containing 1% Triton X-100 and 20% glycerol, and was subsequently incubated for 16 h at 37 °C with or without 2 units *N*-glycosidase F.

proteins from 293-rGC-C cells. The sizes are essentially the same as those observed for human GC-C expressed in the same cultured cell (26). In some experiments a weak band at 90 kDa was also observed (Fig. 3*A*). In mock-transfected 293 cells, no specific proteins were precipitated (not shown). Both the 140- and 160-kDa proteins, as well as an 80–90-kDa band in some experiments, were recognized by the antiserum Z659 when the immunoprecipitated samples were subsequently analyzed on immunoblots (not shown, *cf.* Fig. 5*A*). This demonstrates that the three bands contain the rGC-C carboxyl-terminal sequence and are therefore likely derived from the rGC-C clone. Since no other (not rGC-C derived) proteins were detected after precipitation in our experiments, it is unlikely that rGC-C is tightly bound to other major cellular proteins in 293 cells.

Both the 140- and 160-kDa forms of rGC-C were bound to the lectin, wheat germ agglutinin (WGA), immobilized on Sepharose, and could be eluted with *N*-acetylglucosamine, suggesting that the two high molecular forms of rGC-C are glycosylated (see Fig. 3*B*). However, the 160-kDa form appeared to be enriched in the *N*-acetylglucosamine eluted fraction of the WGA-Sepharose in comparison to the 140-kDa form. The extent of glycosylation of rGC-C was further tested by incubation of the immunoprecipitated proteins with *N*-glycosidase F, which specifically cleaves *N*-linked glycans between asparagine and the carbohydrate chain. As shown in Fig. 3*C*, *N*-glycosidase F converted both the 160- and 140-kDa form to a single 120-kDa protein. The 120-kDa form of rGC-C is presumably the nonglycosylated core protein, as it has almost the same mass as the mature unprocessed rGC-C, calculated from the amino acid sequence (approximately 121 kDa).

The processing of *N*-linked glycoproteins can be impaired at several stages by specific inhibitors (27). Tunicamycin is known to inhibit the attachment of the precursor carbohydrate moiety to the polypeptide chain, causing the expression of proteins missing *N*-glycans. Incubation of 293-rGC-C cells with a relatively high dose (20 μM) of tunicamycin resulted in the subsequent specific immunoprecipitation of a single 120-kDa band representing the non-*N*-glycosylated rGC C (Fig. 4). After treatment of 293-rGC-C cells with a lower dose of tunicamycin, which apparently did not completely block the glycosylation, rGC-C appeared as an array of multiple bands. These bands most likely represent forms of rGC-C to which different numbers of *N*-linked carbohydrate groups are attached. The number of distinguishable bands suggests that most of the 8 consensus *N*-glycosylation sites in the rGC-C sequence (9) are indeed linked to a sugar group. Deoxymannojirimycin and swainsonine are inhibitors of glucosidases involved in the trimming of the initially attached mannose-rich carbohydrate group, thus preventing the subsequent formation of complex carbohydrate structures. Deoxymannojirimycin incubation leads to the synthesis of the so-called high mannose type of glycoproteins, whereas swainsonine allows the processing to continue up to the synthesis of a hybrid type of glycoprotein (27). As shown in Fig. 3, only the 140-kDa protein was visible after deoxymannojirimycin treatment, indicating that this form is most likely the incompletely processed high mannose form of rGC-C. The immunoprecipitation of a protein of approximately 145 kDa with an intensity similar to the 160-kDa protein together with the 140-kDa protein after incubation with swainsonine, but not deoxymannojirimycin, suggests that the hybrid glycosylated rGC-C runs slightly slower than the high mannose type. The absence of the 160-kDa form after incubation with the various glycosylation inhibitors make it very likely that the 160-kDa protein is the completely processed complex type of *N*-glycosylated rGC-C. It is also known for other proteins (*e.g.* epidermal

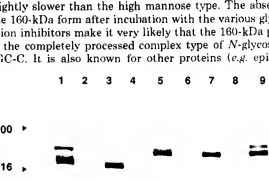

FIG. 4. **The effects of glycosylation inhibitors on rGC-C.** 293-rGC-C cells were labeled with [^{35}S]methionine in the presence of tunicamycin (*tun*), 1 μg/ml (*lanes 1* and *2*); tunicamycin, 20 μg/ml (*lanes 3* and *4*); swainsonine (*sw*), 2 μg/ml (*lanes 5* and *6*); deoxymannojirimycin (*Dmn*), 50 μg/ml (*lanes 7* and *8*); or no inhibitor (*lanes 9* and *10*). The inhibitors were added 2 h before the addition of the [^{35}S]methionine. After solubilization, proteins were immunoprecipitated with anti-GC-C antiserum (*lanes 1, 3, 5, 7,* and *9*) or with antiserum preabsorbed to the GC-C-specific peptide (*lanes 2, 4, 6, 8,* and *10*).

growth factor receptor) that the fully processed protein runs at a higher position than its precursors on SDS-PAGE gels (28).

Since antiserum Z659 was capable of specifically recognizing rGC-C on immunoblots, we used this method to further characterize rGC in isolated 293-rGC-C membranes, and in membranes from rat intestinal epithelial cells. Although the antiserum stained many proteins in 293-rGC-C membranes, only the 140- and 160-kDa bands, as well as an 80–85-kDa band (in some experiments), were stained specifically (*i.e.* blocked by inclusion of the specific peptide)(see Fig. 5*A*). In immunoblots of mock-transfected 293 membranes these specific bands were never observed, although the nonspecific bands were still detected (not shown). This indicates that in isolated membranes the higher molecular weight forms of rGC-C are predominant. Even after a 10-min incubation of the membranes in the presence or absence of STa at 37 °C under the conditions of guanylyl cyclase assays, no change in the intensity of the 140- or 160-kDa bands were observed (Fig. 6), suggesting that the high molecular forms of rGC-C are principally responsible for the observed STa-stimulated cyclase activity seen in 293-rGC-C membranes. However, when rat intestinal brush border membranes, the richest source of intestinal guanylyl cyclase (29), were probed with the anti-rGC-C antiserum, the major specifically stained protein had molecular mass of approximately 85 kDa, although specific bands at 140 and 65 kDa also could be observed (Fig. 5*B*). The intensity of the 65-kDa band relative to the 85-kDa band varied among different brush border membrane preparations from less than 10% to a similar intensity. The 140-kDa protein was relatively enriched in homogenates of intestinal cells (Fig. 5*C*), and it was still visible after treatment of the homogenate in a manner similar to that used for the brush border membrane isolation. However, it was present mainly in the non-brush border membrane fractions (mainly consisting of endoplasmic reticulum and basolateral membranes), and probably represents the incompletely processed form of rGC-C (results not shown). To assure that the lower molecular proteins in brush border membranes were immunologically related to rGC-C, we purified the antiserum Z659 by absorption to, and subsequent elution from immunopurified rGC-C. As shown in Fig. 5*C*, the 85-kDa and, in some experiments, the 65-kDa brush border proteins could still be detected with the purified antiserum directed against the carboxyl terminus of rGC-C. Similarly, the antiserum purified by elution from the 85-kDa band of brush border membranes specifically recognized 140- and 160-kDa bands in 293-rGC-C membranes, as well as the 65-kDa band in brush border membranes (not shown). The 85- and 65-kDa proteins in rat brush border membranes, therefore, are most likely carboxyl-terminal fragments of rGC-C. Further support for this notion comes from comparison of 293-GC-C and brush border membranes on immunoblots under nonreducing conditions (see Fig. 6). When 293-GC-C samples were run on SDS-PAGE in the absence of β-mercaptoethanol, the 140- and 160-kDa bands were absent, and instead specific proteins of approximately 215 and 240 kDa were observed, suggesting that the mobility of rGC-C is different under nonreducing conditions. Incubation in the presence of STa decreased the mobility of these GC-C forms even further (approximately 245 and 275 kDa; as estimated from immunoblots of 5% polyacrylamide gels). The small STa-induced shift in mobility could be caused by a change in the conformation of GC-C, and is probably not related to the STa-induced inactivation of GC-C activity, as the presence 1 mM ATP during the incubation did not prevent the shift in mobility (not shown). Similarly, in brush border membranes analyzed in the absence of β-mercaptoethanol, a protein with a molecular mass of approximately 230,000, but not the 85-kDa protein, was specifically recognized by the GC-C antiserum. The 230-kDa protein also demonstrated a decreased mobility in samples treated with STa. This suggests that GC-C is present in brush border membranes, and that its proteolytic fragments are still linked by inter/intra-molec-

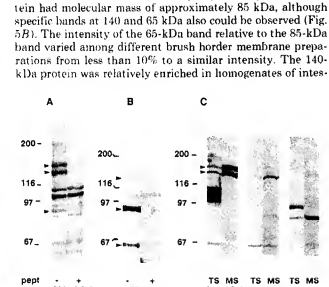

FIG. 5. **Detection of GC-C on immunoblots of 293-rGC-C cells or rat intestinal membranes.** *Panel A*, membranes of 293-GC-C cells (20 μg of protein) were probed with anti-GC-C serum or with antiserum preabsorbed with the GC-C-specific peptide. *Panel B*, rat intestinal brush border membranes (*BBM*) (40 μg of protein) were probed with anti-GC-C serum or with antiserum preabsorbed with the GC-C-specific peptide. *Panel C*, membranes of 293-rGC-C cells (*293-GC-C*), homogenates from isolated rat intestinal epithelial cells (*int. cell*), and rat intestinal brush border membranes (*BBM*) (20 μg of protein each) were analyzed on immunoblots in the same experiment with total anti-GC-C antiserum (1:1000) (*TS*) or with a monospecific antibody eluted from immunopurified rGC-C (*MS*). GC-C was immunoprecipitated from solubilized 293-rGC-C membranes (1 mg of protein), and subsequently incubated on immunoblots with anti-GC-C antiserum (1:300). The antibody was eluted from strips of the blots containing GC-C (between 120 and 180 kDa) by incubation at pH 2.4 as described under "Experimental Procedures." Detection of immunostained bands by the enhanced chemiluminescence technique in case of the MS-treated blots was enhanced by using a 10-fold longer exposure time compared with TS-treated blots. *All panels, arrowheads* indicate immunoreactive bands that were competed away by the GC-C-specific antigenic peptide.

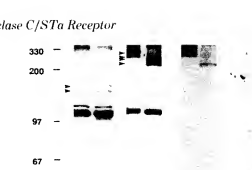

FIG. 6. **Different mobility of GC-C under reducing and nonreducing conditions.** Membranes of 293-GC-C cells or rat intestinal brush border membranes (*BBM*) (200 μg of protein) were incubated for 6 min at 37 °C in 70 μl of 100 mM NaCl, 50 mM Tris/HCl, pH 7.4, in the presence or absence of 1 μM STa. The incubation was stopped by addition of 35 μl of SDS-PAGE sample buffer with or without 70 mM β-mercaptoethanol (*β-ME*), and the samples (20 μg of protein) were subsequently analyzed on immunoblots with anti-GC-C serum (1:1000) as described under "Experimental Procedures." *Arrowheads* indicate proteins which were specifically recognized by the anti-GC-C antiserum.

ular disulfide bridges. It is as yet unclear whether the higher M_r species of GC-C represent dimers of GC-C with faster than expected mobility, or monomers of GC-C that migrate considerably slower compared to monomers in a reduced state. The apparent increase in intensity of the GC-C-specific immunoreactive bands in the absence of β-mercaptoethanol and especially for the brush borders after incubation with STa (see Fig. 6) was consistently found and may be caused by an improved recognition of GC-C in a nonreduced/STa-bound state by the antibody or by a difference in the amount of immunoreactive material in the bands due to a variation in aggregation of GC-C in the samples or a variation in blotting efficiency caused by the different treatments.

The presence of lower M_r carboxyl-terminal fragments of GC-C in intestinal membrane preparations implies, that an NH_2-terminal domain of rGC-C, containing the putative STa-binding site, also should have a lower M_r compared to "full-length" rGC-C. This is the observation in cross-linking studies using ^{125}I STa (8, 12, 13). STa receptors in rat brush border membranes with molecular masses ranging from 55 to 80 kDa are observed and most if not all of them are now more readily explained as fragments of rGC-C, as opposed to products of separate genes. Chemical cross-linking studies with COS-7 cells expressing rGC-C (not shown), or 293-rGC-C, have revealed STa-binding proteins with molecular masses ranging between 45 and 80 kDa, demonstrating that low molecular mass STa receptors are related to the expression of rGC-C (see Fig. 7). Similar low M_r STa-binding proteins have been observed in 293 cells expressing human GC-C (26).

A specific consensus site for enterokinase cleavage (DDDR; amino acids 472–476) close to the putative membrane spanning domain in rGC-C offers one possible target site for proteolysis (30). A proteolytic cleavage of rGC-C in intestinal membranes also explains the finding of Kuno *et al.* (7, 8) that STa receptors and intestinal guanylyl cyclase could be purified in the presence of the reducing agent dithiothreitol as separate proteins, without requiring the assumption that rGC-C is not responsible for the particulate guanylyl cyclase and STa receptor activity observed in intestinal epithelium, in spite of the great abundance of rGC-C mRNA levels in this tissue (9). The brush border enzyme sucrase-isomaltase is known to be proteolyzed after insertion into the apical membrane but to retain function (31). It is therefore conceivable that the cleavage of rGC-C occurs in native brush border membranes as well as after tissue homogenization. Brush border membranes isolated in the presence of 1 mM ATP or prepared from snap-frozen intestine in the presence of 0.5 mM phenylmethylsulfonyl fluoride and 20 μg/ml leupeptin, still contained mainly the 85-kDa GC-C fragment, supporting the former possibility. As judged by the 6-fold higher specific activity of GC activity in brush border membranes (isolated in the presence of 1 mM ATP to minimize inactivation) compared to freshly isolated intestinal cells (26 ± 5 and 4.4 ± 0.5 pmol of cGMP/min/mg of protein, respectively, in the absence of STa and 195 ± 52 and 32 ± 4 pmol of cGMP/min/mg of protein, respectively, in the presence of 1 μM STa; n = 3; see "Experimental Procedures"), the proteolytic cleavage of GC-C does not seem to impair the functional coupling of the STa receptor to the cyclase domain in rat intestinal membranes.

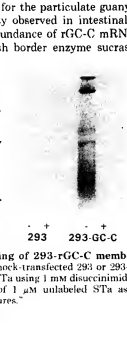

FIG. 7. **Cross-linking of 293-rGC-C membranes with ^{125}I-STa.** Membranes of mock-transfected 293 or 293-rGC-C cells were cross-linked with ^{125}I-STa using 1 mM disuccinimidyl suberate, in the absence or presence of 1 μM unlabeled STa as described under "Experimental Procedures."

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EXHIBIT 4

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Induction of Heat-stable Enterotoxin Receptor Activity by a Human Alu Repeat*

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The heat-stable enterotoxins (ST) elaborated by enterotoxigenic *Escherichia coli* are a family of small cysteine-rich peptides that bind to specific epithelial receptors in the mammalian intestine, causing a secretory diarrhea. The expression of ST receptors is tightly regulated; they are found primarily in intestine, and their expression is developmentally modulated. One receptor for ST has been cloned, and its cDNA encodes a ~120-kDa particulate guanylyl cyclase (guanylyl cyclase-C). Recent studies suggest that there are additional ST receptors that are not homologous to guanylyl cyclase-C. We used an expression cloning strategy to identify intestinal mRNAs that lead to expression of ST receptor activity in transfected cells. Using an ST-specific affinity panning system, we identified a novel 1891-base pair cDNA that does not encode a receptor protein, but instead, consists primarily of untranslated sequence. This cDNA induced receptor activity in both COS and 293 embryonic kidney cells. Northern analysis of the T84 human intestinal cell line, from which this cDNA was cloned, suggests that it is part of a 7.8-kilobase mRNA transcript. This transcript was also identified in human small intestine and colon, as well as in several extra-intestinal tissues. Functional analysis of subcloned fragments reveals that ST binding activity is induced by a 457-base pair human Alu repetitive sequence within the cDNA and that the phenoytpe is independent of orientation. These findings suggest that a human Alu element induces expression of a unique ST receptor by a *trans*-acting mechanism. An unrelated Alu-rich genomic clone did not confer ST binding, suggesting that there may be structural and functional specificity within individual Alu sequences.

Alu sequences are 300-bp[1] repetitive elements that comprise ~6% of the human genome, where they are interposed between single copy genes. They are also present in the untranslated regions of ~10% of hnRNAs and mRNAs (1). Despite the abundance of Alu sequences, surprisingly little is known about their biological effects. There is some evidence implicating Alu sequences as regulators of cellular differentiation, and of developmental and tissue-specific gene expression (2–6).

The heat-stable enterotoxins (ST) are a family of small cysteine-rich peptides that are secreted by enterotoxigenic *Escherichia coli*, a major etiologic agent of diarrhea for travelers and infants in the Third World. These peptide toxins bind to specific intestinal receptors. The toxin-receptor interaction causes increased chloride secretion, resulting in a secretory diarrhea (7). Recently, the mammalian intestinal peptide guanylin has been isolated and its cDNA cloned (8–10). The amino acid sequence, receptor specificity, and pharmacologic properties of guanylin are similar to ST, which suggests that guanylin is an endogenous ligand for ST receptors.

Although the physiologic role of ST receptors is unknown, their expression is tightly regulated. ST binding sites are sparse in intestinal crypt cells; they increase in abundance as these proliferating crypt cells become terminally differentiated villus enterocytes (11–13). ST receptors are also developmentally regulated, with maximal expression in embryonic and early infant life (14). The molecular basis for this cell specific and developmental expression has not yet been determined.

One ST receptor has been cloned, and its cDNA encodes a ~120-kDa particulate guanylyl cyclase (GC-C) (15, 16). However, biochemical studies of brush-border membranes suggest that there may be multiple intestinal ST-binding proteins, ranging in size from 45 to 160 kDa, some of which lack guanylyl cyclase activity. Some of these lower molecular weight species may be post-translationally processed fragments of GC-C (17, 18). Other ST-binding proteins may represent unique classes of receptors (19–21) that are not homologous to GC-C (22). This hypothesis is especially relevant, because another member of the particulate guanylate cyclase family (atrial natriuretic peptide receptor-A, molecular mass 130 kDa) has a cyclase independent, nonhomologous counterpart (atrial natriuretic peptide clearance receptor) that is a 70-kDa protein (23).

In the study described below, we used an expression cloning strategy to identify mRNAs that either encode novel ST receptors, or induce ST receptor expression in transfected cells. A unique 1.9-kb cDNA that induces ST binding activity when transfected into COS and human embryonic 293 cells was cloned and characterized. We show that the ST binding phenotype can be conferred by an Alu sequence within this cDNA. These findings demonstrate that an Alu sequence can induce expression of ST receptor activity and may do so by a *trans*-acting mechanism.

EXPERIMENTAL PROCEDURES

cDNA Library—The T84 human colon carcinoma cell line was obtained from ATCC and maintained as previously described (12). Total RNA was prepared from ~70% confluent cells (24); poly(A) RNA was then selected with two rounds of oligo(dT) chromatography. First strand synthesis was performed using 5 μg of poly(A) RNA, oligo(dT) primers, and avian myeloblastosis virus reverse transcriptase. After second strand synthesis, the resulting cDNA was ligated (25) to *Bst*XI adapters

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L20117.

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[1] The abbreviations use f are: bp, base pair(s); ST, heat-stable enterotoxins; GC-C, guanylyl cyclase C; kb, kilobase pair(s); nt, nucleotide; BST, biotinylated ST1b; *HSAG-1*, human surface antigen-1; ST1b, heat-stable enterotoxin 1b; Alu, Alu repetitive sequence.

(Invitrogen, San Diego, CA). cDNAs >1.5 kb were size-selected by agarose gel electrophoresis and purified by electroelution. The SV40-based eukaryotic expression vector pCEV4 was digested with *Bst*XI to remove the 450-bp stuffer (26). The cut vector was gel purified, then ligated to the cDNA. The ligation products were then used to transform *E. coli* WM1100 by electroporation, and a library of 1.3×10^6 recombinants was prepared.

Plasmid Construction—Constructs were cloned in *E. coli* WM1100 using either vector pCEV4 (26) or pME18S, both of which have the same SV40 promoter (SR-α), but differ in their polylinkers. These vectors were kindly provided by Dr. A. Miyajima (DNAX, Palo Alto, CA). Constructs (shown in Fig. 4) were characterized by restriction mapping, and in some cases, by partial sequencing.

Plasmid pS604 contained nt 1–604 and was inserted into pME18S with the Met (position 449) of the potential open reading frame in the same orientation as the SV40 promoter. It was prepared by excising this fragment from Bluescript KSII+ using *Pvu*II and *Xba*I. The fragment was directionally cloned into the filled-in *Eco*RI site and the *Xba*I site of pME18s. Plasmid pS1891 contained nt 605–1891, and was prepared by digesting clone 3 with *Pvu*II-*Pst*I, and ligating the fragment to the filled-in *Eco*RI and *Pst*I site of pME18S. Plasmids pS1679 (forward and reverse orientations) were prepared by digesting pS1891 with *Eco*RI, and ligating the fragment corresponding to nt 605–1679 (of clone 3) into the *Eco*RI site of pME18S. Plasmid pS1243 (nt 1243–1891) was prepared by polymerase chain reaction. The forward primer corresponded to nt 1243–1263 with an added 5' *Bam*HI site (5'-GTACGGATCCCGGCGCTGTACAITTGCATTCCTG). The reverse primer (5'-ATCAGCTTGGGCCTAGCAGT) was complementary to vector pCEV4 just 3' to the *Pst*I site. This fragment was amplified from clone 3, digested with *Bam*HI-*Pst*I, and ligated to pCEV4 (*Bam-Pst* sites).

Plasmid pS1237 (nt 605–1237) was prepared by digesting pS1891 with *Sna*BI, filling in, and then cutting with *Eco*RI. This fragment was directionally cloned into the *Eco*RI and filled-in *Pst*I sites of pME18S. Plasmid pS1062 (nt 605–1062) was prepared by gel purifying this 457-bp fragment (*Pvu*II-*Bf*aI) from clone 3, filling in, then ligating to the filled-in *Xho*I sites of pME18S.

Transfections and Affinity Panning—Peptide toxins ST1b (ST) and biotinylated ST1b (BST) were synthesized as previously described (12). COS-7 cells ($\sim 1.3 \times 10^6$) and 293 cells ($\sim 5 \times 10^6$) were transfected with 10 μg of clone 3, or equimolar amounts of other clones using DEAE-dextran (12). Human 293 embryonic kidney cells were obtained from the laboratory of Dr. Brian Kobilka (Stanford University Medical Center). They were transfected under the same conditions as described for COS cells with the following modification. Cells were incubated with DNA, 10% fetal calf serum in Dulbecco's modified Eagle's medium, DEAE-dextran, and chloroquine for 2 h as described by Conklin *et al.* (27). They were then transferred to a 30-ml conical tube, where they were treated with phosphate-buffered saline containing 10% dimethyl sulfoxide followed by three phosphate-buffered saline washes. Cells were then replated in 10-cm Falcon dishes and harvested at 72 h. These modifications were necessary because 293 cells are more susceptible than COS cells to detachment during transfection.

Affinity panning assays were done using Petri plates coated with affinity purified anti-biotin (Sigma) as previously described (12). The clone GC-C-pSVL was generously provided by S. Schulz and D. L. Garbers (University of Texas, Southwestern), and *HSAG-1*-pSV$_2$-neo was kindly provided by C. P. Stanners (McGill University). ^{125}I-STa radioligand binding and radioimmunoassays for cGMP were performed on transfected COS cells as previously described (12, 16, 28).

Northern Analysis—Total RNA was prepared from COS-7 cells 72 h after transfection using RNAzol (Tel-test, Friendswood, TX). Poly(A) RNA from T84 cells was prepared as described above. RNA was separated on a 1.3% formaldehyde/agarose gel which was then treated with 0.05 N NaOH. After neutralization (20 $\times$ SSC), the material was capillary blotted to uncharged nylon and cross-linked. The 5' probe (nt 1–517) was prepared by digesting a clone 3 at the *Xho*I-*Bst*YI sites and the 3' probe (nt 1243–1891) was prepared by digesting pS1243 at the *Bam*HI-*Pst*I sites. Hybridization was performed at 65 °C (2.5 h), using Rapid Hybridization Buffer (Amersham); blots were then washed at a final stringency of 0.2 $\times$ SSC, 0.2% SDS at 60 °C. Human poly(A) RNA from spleen, testes, prostate, ovary, small intestine, and colon was electrophoresed (2 μg/lane) on a 1.2% formaldehyde/agarose gel, and transferred to nylon (Multiple Tissue Northern-II, Clontech, Palo Alto, CA). Each lane contained an equivalent signal for B-actin expression. The membrane was probed and washed under the same stringent conditions as described above.

RESULTS

In order to identify novel ST receptors or genes inducing ST receptor expression, we used an SV40 based expression cloning strategy (25, 29). COS cells were transfected with a human intestinal cDNA library from the T84 cell line, a human colon carcinoma-derived cell line that expresses ST receptor activity. The transfected COS cells were then subjected to affinity panning using BST, a biotinylated derivative of ST, the preparation and pharmacologic properties of which we have previously described (12). Transfected cells expressing ST receptor activity were then captured onto plastic coated with an antibiotin antibody. Plasmids from the captured cells were extracted, and the process of transfection followed by affinity panning was repeated several times, thereby creating an enriched cDNA pool (25, 29). Ligand-specific capture of cells was ascertained by lack of adhesion in the presence of biotin alone or ST alone, and by competitive inhibition studies using ST.

Identification of a cDNA Clone Exhibiting ST Receptor Activity—Three iterative cycles of panning and transfection were performed (25). After the first cycle, <1% of cells were adherent to antibiotin-coated plates. When panning was done with the enriched plasmid pools (*i.e.* second and third cycles), ~20–30% of COS cells were adherent to the antibiotin-coated plates (not shown). Several clones from the third cycle, with inserts ranging in size from 2 to 4 kb, were selected from the enriched pool and individually tested for ST binding activity.

Three of these clones were tested by the ST-specific affinity panning assay described above. Clones I and 2 exhibited negligible adhesion (not shown). In contrast, clone 3 showed marked ST-specific adhesion (38.5 $\pm$ 1.3% S.E., where n = the number of plates analyzed, Fig. 1). Its adherence was similar in magnitude to transfectants expressing GC-C-pSVL (guanylyl cyclase-C cDNA in the SV40 based expression vector pSVL), the positive control (34.3 $\pm$ 3.7%) (12). These values are consistent with the transfection efficiency of ~35–45% that we obtain using pCH110, an SV40 based expression vector encoding lacZ (12). The adherence of clone 3 transfectants was ~15-fold greater than the level of adherence seen with the transfected vectors pCEV4 and pSVL (without insert), which were used as negative controls. Adherence was ST specific in that it was reduced to 3.0 $\pm$ 0.6% in the presence of excess unlabeled ST (Fig. 1), but not affected by equimolar concentrations of an unrelated ligand, atrial natriuretic peptide (α human 1–28) (33.0 $\pm$ 0.8%, n = 4; not shown). Furthermore, adhesion did not occur in cells transfected with clone 3, if BST was replaced with native ST or with biotin (<1%, not shown).

To further validate these findings, clone 3 was also transfected into human embryonic kidney 293 cells as described under "Experimental Procedures." Like COS cells, 293 cells are easily transfected with DEAE-dextran, and do not express endogenous ST receptors. Data using the ST-specific affinity panning assay are shown in Fig. 1*B*. Human 293 cells transfected with clone 3 showed BST-dependent adhesion to antibiotin plates (43.5 $\pm$ 3.8% S.E.), and this adhesion was markedly inhibited by the presence of excess unlabeled ST (9.3 $\pm$ 4.0%). 293 cells transfected with GC-C (positive control) were adherent (54.3 $\pm$ 2.4%) when incubated with BST, and this adherence was also inhibited in the presence of excess unlabeled ST (5.3%, n = 2). Vector transfected 293 cells (negative controls) showed minimal BST-dependent adherence (6.9 $\pm$ 2.5%). These findings demonstrate that ST receptor activity is inducible in human (293) as well as in monkey (COS) cells.

Six clones obtained from the enriched library, including clone 3, were individually transfected. The transfected cells were tested for ST-receptor activity using ^{125}I-STa in a radioligand filter-binding assay (12, 28). No specific binding was detected in

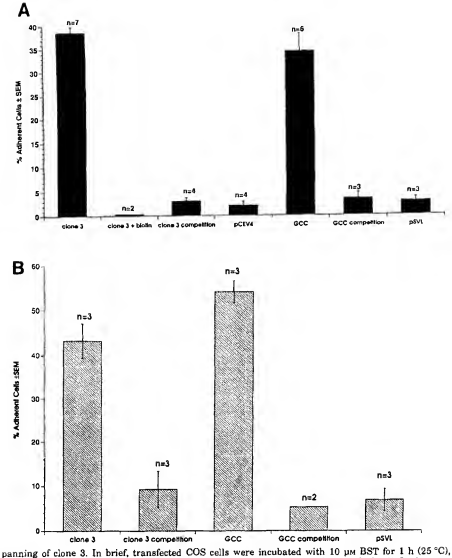

FIG. 1. *A*, affinity panning of clone 3. In brief, transfected COS cells were incubated with 10 μM BST for 1 h (25 °C), washed, seeded onto antibiotin-coated plates, and the percent adherent cells determined after washing as previously described (12). In the experiment labeled *clone 3 + biotin*, 10 μM biotin was substituted for BST. In experiments labeled "competition," the cells were preincubated for 30 min with 3 μM ST1b followed by addition of BST (10 μM), and incubation continued for 1 h. pCEV4 corresponds to cells transfected with vector. *N* represents the total number of plates analyzed, and *error bars* show the S.E. for all plates tested with a given clone. GCC corresponds to COS cells transfected with clone GC-C-pSVL. GCC competition was carried out by incubating transfected cells with excess unlabeled ST1b as above. pSVL is the SV40 expression vector without insert, into which GC-C-pSVL was cloned. The three experiments with GC-C-pSVL have been previously published (12), and are presented here as a positive control. *B*, human embryonic kidney 293 cells were transfected using DEAE-dextran with chloroquine as described under "Experimental Procedures." Affinity panning experiments were performed as described in *A*.

these transfectants, in contrast to GC-C-pSVL transfectants, which exhibited ~10% specific binding. Cells transfected with the positive control GC-C-pSVL showed a ~25–50-fold increase in intracellular cGMP (16) in the presence of 4 μM BST or ST1b. In contrast, clone 3 showed no guanylyl cyclase stimulation in response to these ligands (not shown). This indicates that although COS cells transfected with either clone 3 or with GC-C will bind ST specifically in the affinity panning assay, the ST binding phenotypes induced by these two cDNAs are different. Affinity panning has been demonstrated to enrich for low affinity interactions through multiplicative binding (30). We hypothesize that transfection with clone 3 induced expression of ST-specific low affinity binding that was detectable by affinity panning, but not by the less-sensitive radioligand binding assay.

The ~2.0-kb insert (clone 3) was used as a probe to identify additional hybridizing species in the T84 cDNA library by Southern blotting (Fig. 2). A prominent band, approximately the same size as the clone 3 insert, was seen in the enriched cDNA library (Fig. 2, *lane 2*), suggesting that iterative panning had enriched for this clone.

Sequence Analysis—The nucleotide (nt) sequence of clone 3 (1891 bp) is shown in Fig. 3*A*, and a schematic representation of its organization is presented in Fig. 3*B*. The sequence orientation is presented with respect to the SV40 promoter of pCEV4. Analysis of this sequence revealed an incomplete open reading frame extending from nt 1 to 449, that was in the reverse complement orientation to the SV40 promoter. The deduced polypeptide sequence of this potential reading frame was interrupted after amino acid 149, where it adjoined the vector. The Kozak consensus sequence neighboring the ATG at nt 449 was unfavorable (31). The hydropathy profile of this putative polypeptide was not suggestive of a transmembrane protein, and it had no significant similarities to protein sequences in

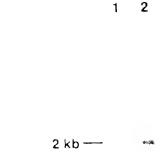

Fig. 2. **Southern blotting of cDNA libraries.** GC-C-pSVL was digested with *Xho*I-*Sal*I, all other DNA was digested with *Xho*I to release inserts. Samples were electrophoresed (1% agarose), capillary blotted to uncharged nylon, and cross-linked (UV-Stratalinker, Stratagene). The 1.9-kb insert of clone 3 was labeled with [^{32}P]dCTP by random priming. Hybridization was performed at 65 °C in rapid hybridization buffer (Amersham); this was followed by washing at a final stringency of 0.7 × SSC, 0.1% SDS (65 °C). *Lane 1* contains 2 μg of plasmid DNA from the T84 cDNA library before selection. *Lane 2* contains 2 μg of DNA from the enriched plasmid library recovered after 3 rounds of panning. *Lane 3* contains GC-C pSVL (1 μg). *Lane 4* contains vector pCEV4 (1 μg). A smear of hybridizing signal was seen in both the *Lanes 1* and 2, but was absent in the lanes containing vector alone (*Lane 3*) or GC-C-pSVL (*Lane 4*). In view of the sequence (Fig. 3), it is likely that this diffuse pattern of lane-specific hybridization is due to the presence of a 628-bp Alu cluster, with a mid-poly(A) tract. Both of these sequence motifs are highly abundant in cellular mRNA.

GenBank. Based on this analysis, we conclude that this open reading frame is unlikely to encode a protein with structural features typical of a cell surface receptor.

Analysis at the nucleotide level revealed that the clone contains an Alu cluster (nt 516–1153). This cluster is comprised of two human-specific Alu repeats from different subfamilies, that are arranged in a nested configuration (Fig. 3*B*). Fig. 3*C* shows both Alu repeats aligned to their consensus sequence in the reverse complementary orientation relative to their original presentation in Fig. 3*A*. The evolutionarily younger of the two, classified as Alu-Sx (661–1007), is flanked by 7-bp tandem repeats (CCAGGTA, Fig 3*A*, *lower case letters*) and contains a 79-bp mid poly(A) tract (Fig. 3*A*). The Alu-Sx sequence is inserted into the middle of the evolutionarily older Alu-J sequence, and splits it into two parts (Alu-J-Right, 516–643, and Alu-J-Left, 1015–1153 (32, 33). The polymerase III split promoters conform to the polymerase III consensus (34). Both the Alu-Sx and Alu-J sequences are mutated to a degree consistent with their respective ages, with the exception of the perfect 79-bp poly(A) region within Alu-Sx, which appears to be preserved without mutation. The lack of mutation in this poly(A) tail was verified by sequencing several times, and may contribute to the biological activity of the Alu sequence described here. Mid poly(A) tails can sometimes be seen in cDNAs that are inadvertently ligated together during library preparation. This is unlikely to be the case here for the following reasons: 1) the Alu fragments flanking the Alu-Sx sequence containing the poly(A) tail belong to the same evolutionary family, *i.e.* Alu-J; 2) Alu-Sx is flanked by short tandem repeats in almost perfect positions at the beginning and end of the Sx sequence indicating its insertion (Fig. 3*A*, *lower case letters*); 3) the younger Alu is inserted into the older one as logically expected, and 4) the sequences conform to the typical Alu consensus (Fig. 3*C*).

Nucleotides 1154–1891 contain AT-rich (66%) sequences for which no significant homologies were found. No other reading frames >200 bp were found.

Functional Analysis of Subclones for ST Binding Activity—In order to determine the sequence requirements needed for expression of the ST-binding phenotype, various regions of clone 3 were subcloned and then tested for ST binding activity using the affinity panning assay described above (Fig. 4). In order to establish whether the ST binding activity was attributable to the partial open reading frame, subclone pS604 was constructed. This construct contains the partial reading frame in the same orientation as the SV40 promoter, plus 85 bp of the right Alu-J monomer. Transfection of pS604 resulted in a small amount of cellular adhesion (6.4 ± 1.8%) that was only slightly greater than the vector background (3.1 ± 0.1%), and markedly less than the full-length cDNA (38.5 ± 1.3%). Next, we tested pS1891 (nt 605–1891), which contains the remaining sequence of clone 3 (most of the Alu cluster as well as the AT-rich 3′ end). Transfectants exhibited ligand-dependent adhesion that was similar in magnitude (33.8 ± 5.6%) to that of the full-length clone (38.5 ± 1.2%). This adhesion was specific in that it was competitively inhibited by unlabeled ST to a background level (3.2 ± 1.3%).

We next asked whether this phenotype was conferred by a more circumscribed region of the noncoding sequences of clone pS1891. To study this, we tested a construct containing the region that was 3′ to the Alu cluster (pS1243 (nt 1243–1891); Fig. 4). Cells transfected with clone pS1243 did not exhibit BST binding activity (3.24 ± 0.7%).

The remaining region to be tested was the Alu cluster. A 632-bp fragment containing nt 605–1237 was subcloned (pS1237). It contained the truncated right Alu-J monomer, the full S dimer, and the complete left J monomer, and in addition, 83 bp of a non-Alu flanking sequence (nt 1154–1237). When transfected into COS cells, pS1237 was found to be highly active, with 33.6 ± 3.4% of cells adherent to antibiotin. Adherence was inhibited by excess unlabeled toxin (6.9 ± 3.1%), indicating that it was ST-specific.

Sequences that flank certain Alu elements have been shown to influence their cellular effects (4). Therefore, a construct containing sequences within the Alu cluster, but excluding flanking sequences was tested. This construct (pS1062, in the same orientation as Clone 3) contained the intact Alu-Sx dimer, flanked on both sides by truncated J monomers. When COS cells were transfected with pS1062, BST binding was preserved and in fact slightly increased (44.6 ± 2.0%) compared to the full-length clone. Ligand-dependent adherence was reduced to 3.6 ± 0.8% by excess unlabeled toxin, again demonstrating specificity for ST. Thus, the Alu sequence was sufficient to induce this phenotype.

Finally, we tested the effect of orientation with respect to the SV40 promoter, on expression of the ST binding phenotype. Two constructs (pS1679 forward and reverse), corresponding to nt 605–1679, were transfected and tested. The cellular adhesion was 37.9 ± 4.3% for construct pS1679-forward, which was in the same orientation as clone 3, and only slightly lower (31.9 ± 3.1%) for the subclone in the opposite orientation (pS1679-reverse).

Transfection of COS Cells with HSAG-1, An Alu-rich Genomic Clone—Having established that an Alu sequence induces this ST-binding phenotype, we tested a different Alu-rich gene known as *HSAG-1* (human surface antigen). This 4.4-kb genomic clone is derived from a human-Chinese hamster ovary

A

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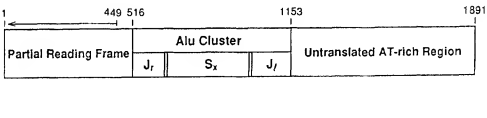
5'  CTCTAAAGGG AAATCCCAAC CTTCTTTGGT CTTGTAGTTT TGAACAGGGA GGGTATCCTG TATTACACCT CCAATACGGC    80
 81 TCAGTCTTAG GAGGAAGCAT TCAAAGTCTT CCAGTTCAGA TGCCAAAAAA ACCCCATTCC TGGCCAGCTT ATCTAAAATT   160
161 TCATTCCTCA TGTAGTTGTT ACAGGAAGTA TTCTGGTTAA TAACATCAAC AGTCAGAAGG GGCCATTTGT TCTGCTGAAG   240
241 TGAAGCATAA CTATGCTGGG TTTGAAATTC TCCAATCCAT AAGATAATGT TTAACCAATC ATGGCGAGCT GTGAGGTATC   320
321 TCCAGAGGGC TTCAGGGGAA TATGATTTGT ATTCTACATG AAAAAAAACA CATTTTAAAA TATAATTAAT TATGTCTATT   400
401 GACATGTAAC TCAACACTAA ATTACTGAGT AAATATAACA ATCTGGACAT AGATATTTAT ACAATATATT AATTATTCAG   480
481 GTCCTCTGTT TTACTATTTT CCTTTTTTTG AGACAGGATC TCGCTGTGTT GCCCAGGCTG GAGTGCAGTG GTGCACTCGT   560
561 GGCAGCCTCG ACCTCCTGGG CTCAAGTGGT ACTCCTACTT CAGCTGCCCA AGTAGCAGGG ACTACAGGGA GTTGCCATCA   640
641 TGAccaggtc AATTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT   720
721 TTTTTCTTTT TGAGACAGAG TCTCGCTCTG TAGCCCAGGC TGGAGTGCAG TGGCGCAATC TTGGCTCACT GCAACCTCTG   800
801 CCTCCCAGGT TCAAGCGATT CTCCTGCCTC AGCCTCCCAA GTAGCTGGGA TTACAGGAAT GCACCACCAC ACCCAGCTAA   880
881 TTGTTCTATT TTTAGTAGAG GCGGGGTTTC ACCATGTTGG CCAAGCTGGT CTTGAACTCA GGTGATCCGC CTGCCTCAGC   960
961 CTCCCAAAGT GCTGGATTA TAGGCGTGAG CCACCACGCC CGTCCAccca ggtaTTTAA AAAATATATA TTCTGTAGAG  1040
1041 ATGGGGTCT CACTATGTTG CTAGGCTGGT CTTGAACTCC TGGCCTCAAG CAATCTTCTC ATCTCGGTTT CCCAAATTTC  1120
1121 TGGGATTACA GGTGTGAGCC ACCGTGCCTG GCCACATTGT TCATTTTATA ATCAATGCTA CAGCCAAAGA ACTAGATGTA  1200
1201 GCTTCTAATT ACTCTGGTGG TTAAAATGAA ACTTACGTAT TAGCTGTACA TTTCACTTAC CTGTTCAACC CTTTTTTTCC  1280
1281 CTTCTCTTTC CCTTAATGTA AATAAAACT TTGTTTCAGA TGGGGAAGGA TGAAAAGAAA AAAATAAATT AAAAATAACA  1360
1361 AACAAACAAA AACTTCAGCC ATAATAGTAA GCCAGAATTT TACTCTCTGA ATTGCTACTG TTTACCACTG TTAGTTATGA  1440
1441 CCCAGATCCC CCATATCAGA TGGCATAGAT CCCCAATTTA CACAAGAAGC TCAAGCTAT TTGAAAAAAC AGATTGTCAC  1520
1521 TTTGAGCTG GAATGAAAAC TCAGCCTTGA AATCTCAATT AACTATTTTT TTCTTTTATT AGCACATTAT AAATCCCTCC  1600
1601 CAGAAACTCT GAGATAAGAA AGAGATCTTA ATGCAACGAC TTCATTTTTA AAGAACCTGA ATATTATTTC CCGAAAGGAA  1680
1681 TTCTAAAATT GAGACAGATC AAAATATTCA AATATCAATA CCCAATTTAA TCCTCATTCA GTATGTTCAT ATTTCAGGCT  1760
1761 CTCTCACACT TGCCTTCTGG ACTTATCCTG GGGAGAAGGA TGGATTCTTG TGTTAGTTGA TCCCACCACA GAGCCCAATT  1840
1841 TAACACAATT CTATGGTCCT GTTTGTTAAA TTCATCTTTA CCTTTAGAGC A  3'
```

B



C

```
          1                                                                                  95
Alu-cons  GGCCGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCGAGGCGGGCGGATCACCTGAGGTCAGGAGTTCGAGACCAGCCTGGCC
Alu-Sx    ..A.....T.....................A.....................T....A.....A.....----------.....
Alu-J(1+z) ....A...A.....................A.....A.....AA....AT.A.AA...TG.T.....C.....A.....A.-.

          96                                                                                 189
Alu-cons  AACATGGTGAAA-CCCCGTCTCTACTAAAAATACAAAAATTAGCCGGGCGTGGTGGCGCGCGCCTGTAATCCCAGCTACTCGGGAGGCTGAGGCA
Alu-Sx    .....T.....-.....C.....................C.....T...T.....T..ATT.....T.....C.....
Alu-J(1+z) ....A...G.C....A.....AG..T...T.TTTT...AAAAAT.A..A....A.ATC.....G...T.....T...CA.....A.T.

          190                                                                                282
Alu-cons  GGAGAATCGCTTGAACCCGGGAGGCGGAGGTTGCAGTGAGCCGAGATCGCGCCACTGCACTCCAGCCTGGGCGACAGAGCGAGACTCCGTCTC
Alu-Sx    .....T.....T.....A.....A....T.....T.....T.....T.....T.....
Alu-J(1+z) ....T.C.A.....G...A....TC....C...CAC...-------T..A.....A...C.....TC.------
```

FIG. 3. **Sequence analysis of clone 3.** *A*, complete nucleotide sequence. Double stranded sequencing was performed (Sequenase 2, U. S. Biochemical Corp.). The sequence was analyzed using IntelliGenetics Suite (IntelliGenetics Corp., Mountainview, CA). The Alu cluster is *underlined*, and flanking repeats are in *lower case letters*. *B*, overview of sequence domains. For the purposes of structure-function analysis, the clone was divided into three general domains. These consist of the 5'-region flanking Alu, which contains a truncated potential reading frame, the Alu cluster, and an AT-rich untranslated region. Alu sequences were human-specific, and consist of an Alu dimer (651–1005, flanked by 7-bp tandem repeats) with a 79-bp mid-poly(A) tract, belonging to the Sx evolutionary family. The dimer is inserted into the middle of the evolutionarily older Alu-J sequence, and splits it into two parts (Alu-J-Right, 516–643 and Alu-J-Left, 1015–1153) (32, 33). *C*, alignment of Alu-Sx and both halves of Alu-J to the Alu consensus. The regions containing "..." depict identity to the consensus. The symbol "-" shows alignment gaps, and "xx" separates the left and right arms of Alu-J.

hybrid cell line, and contains multiple Alu-like sequences; some of these repeats are rodent specific, and others have homology to human Alu (J-family). *HSAG* has been shown to induce expression of a leukemia-associated cell surface antigen in stable transfectants, and also to inhibit cell proliferation when transfected into HeLa cells (35–37). It was therefore of interest to test the biologically active *HSAG* clone in our system, to address the question of whether other Alu sequences could also induce ST binding activity. *HSAG-1* (under the same SV40 promoter as GC-C-pSVL, Fig. 1) was transfected into COS cells, and the transfectants tested for ST receptor activity by affinity panning. The transfected cells showed negligible adhesion (1.5 ± 0.7%, $n = 5$) to antibiotin after incubation with BST.

Northern Analysis of Transfected Cells, the T84 Cell Line, and Human Tissues—In order to study the expression of clone 3-specific RNAs in transfected COS cells, probes corresponding

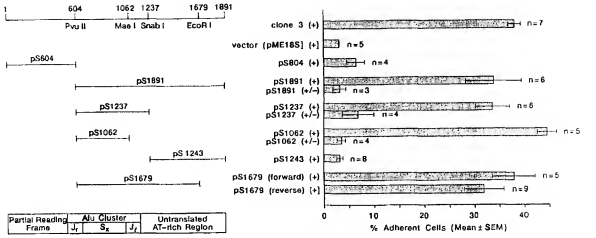


FIG. 4. **Affinity panning with subcloned fragments of clone 3.** Transfections and panning were performed as described in the legend to Fig. 1 using plasmids containing various fragments of clone 3. Transfections were performed at least twice for each plasmid construct; *n* represents the total number of plates analyzed. Constructs shown with the symbol (+) represent transfectants incubated with BST alone. The symbol (+/–) represents a competitive inhibition control containing saturating concentrations of ST in the presence of BST.

to the regions directly flanking Alu were prepared. These probes contained the non-Alu flanking sequences of clone 3 (nt 1–517, 5′ probe and nt 1243–1891, 3′ probe). Although these flanking sequences do not confer receptor activity to transfected cells, they were used for Northern analysis in order to avoid nonspecific hybridization with the many Alu-like sequences that are present in human RNA (Fig. 2). As expected, Fig. 5*A* (5′ probe) demonstrates that COS cells transfected with clone 3 produce an abundant ~2.2-kb transcript that is easily detectable in total RNA (*Lane 2*). This is consistent with RNA polymerase II transcription of the cDNA insert originating at the SV40 promoter of pCEV4. No hybridization was detected in COS cells transfected with vector alone (*Lane 1*).

Fig. 5*B* shows a Northern blot containing 5 μg of poly(A) RNA from the T84 cell line, probed with the 5′-flanking sequence. A single ~7.8-kb transcript was detected, suggesting that clone 3 is part of an expressed message in the T84 cell line. The transcript could not be detected in 35 μg of total RNA from T84 cells (not shown), indicating that it is of relatively low abundance. Fig. 5*C* demonstrates that there is a single ~7.8-kb transcript detectable in poly(A) RNA from other human tissues including spleen, thymus, testes, ovary, small intestine, and colon (3′-flanking probe). The 5′ probe showed faint bands in this same region of the human tissue blot (not shown). In contrast, when this human tissue blot was reprobed with GC-C-specific sequences, transcripts (~1 kb) were detected only in small intestine and colon (not shown), consistent with previously published work (15).

DISCUSSION

In this report, we describe a novel cDNA from the T84 human intestinal cell line that induces heat-stable enterotoxin binding activity in transfected COS cells and in human embryonic kidney 293 cells. The phenotype seen in these transfected cells fullfills criteria for receptor activity in that it is ST-mediated, and specifically inhibited by excess unlabeled ligand. Furthermore, incubation of transfected cells with biotin alone, or with unlabeled toxin does not promote their adherence in this affinity panning system. Blocking the immobilized antibody with biotin abolishes this ligand-dependent cellular adhesion. Taken together, these data indicate that transfection of cells with this cDNA leads to the expression of specific ST binding activity by the transfected cells. By testing subcloned fragments of the cDNA, we established that a 457-bp fragment composed of Alu repetitive sequences within the cDNA is sufficient to induce expression of this phenotype, and that regions of the cDNA that did not contain Alu were inactive. This Alu sequence is somewhat unusual; it is a cluster of two nested Alu dimers of different evolutionary ages containing a mid-poly(A) tract that is preserved without mutations expected in Alu sequences of this evolutionary age. The locations of both the poly(A) tract and the flanking repeats of Alu conform to the consensus. This supports the view that this is a naturally occurring sequence, and raises the possibility that the mid-poly(A) tract may be a conserved region.

This cDNA was selected by affinity panning. The ST-binding phenotype of the clone reported here was the same as the GC-C receptor, as determined by the affinity panning assay used here. However, it differs from GC-C in that it neither induces specific binding to ^{125}I-STa, nor activates guanylyl cyclase in response to toxin. Because the panning assay entails multiplicative ligand-receptor interactions, it is inherently more sensitive than solution-phase radioligand binding. Thus, it is likely that our use of iterative panning led to selection of a low affinity ST binding phenotype, that was not detectable with radioligand binding, but only detectable in the more avid affinity panning system. Indeed, it has been demonstrated that affinity panning strongly selects for low affinity interactions (30). Many ligands, including nerve growth factor, interleukin-7, and peptide-major histocompatibility complexes participate in biologically important low-affinity, ligand-specific, receptor-mediated events (38–40). Recently low-affinity cyclase-independent ST binding sites have been identified in an intestinal crypt cell line (22); this activity may be relevant to the phenotype described here.

A concern about the Alu-induced phenotype reported here is that it has been identified using transient expression systems, where transfected plasmids containing SV40 promoters are replicated to high copy number, and could potentially cause nonspecific effects. To address this issue, we transfected COS cells with *HSAG-1*, an Alu-rich genomic clone (driven by the same SV40 promoter as the GC-C-pSVL clone), and it did not induce ST binding. Although the high level of replication of clone 3 in transfected COS cells may have enhanced detection of ST binding, the negative result with *HSAG* indicates that there may be structural and functional specificity within individual Alu elements.

Northern analysis of COS cells transfected with clone 3 demonstrates the presence of an abundant ~2-kb RNA transcript,

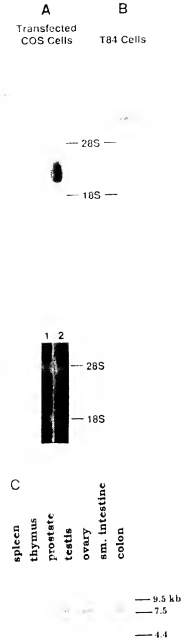


FIG. 5. Northern analysis of transfected COS cells, the T84 cell line, and human tissues. *A* and *B* were analyzed with a ^{32}P-labeled 5′-flanking region of clone 3 nt 1–517. Fig. 3). Both lanes in *A* contain 5 μg of total RNA from COS cells transfected with either vector pCEV3 without insert. *Lane 1*, from clone 3 in pCEV3; *Lane 2*, 2-h exposure. Below, RNA loaded into these lanes is visualized with ethidium bromide. The blot in *B* contains 5 μg of poly(A)$^+$ RNA from T84 cells. 5-h exposure. *C* shows a blot containing 2 μg of human poly(A)$^+$ RNA per lane. Hybridization was performed using the 3′ region (nt 123–581 flanking Alu). 16-h exposure.

as other human tissues, suggests that it is part of a 7.5-kb mRNA transcript (Figs. 5, *B* and *C*). This 7.5-kb mRNA was found in spleen, thymus, prostate, ovary, and small intestine and colon (Fig. 5*C*). This is in contrast to the 4 kb GCC transcript, which is found exclusively in small intestine and colon (Fig. 5*B*). Differences in the expression of these two mRNA species are not surprising considering that 1) the nucleotide sequences of clone 3 and of GCC are unrelated, and that 2) the pharmacological properties of cells transfected with clone 3 are distinct from those transfected with GCC. Furthermore, unlike GCC the Alu sequence does not itself encode a receptor protein, but instead induces expression of ST binding activity. From these observations we hypothesize that this Alu sequence might regulate other cellular functions in tissues that do not express ST binding activity.

Other Alu-rich mRNAs have also been associated with specific cellular effects. Garret *et al.* (14) describe a gene that induces expression of human interferon-inducible protein 9-27. This gene had no significant open reading frames but contained many Alu-rich regions. Similarly, Kageyama *et al.* (15) identified 12 unique mRNAs that were specifically expressed in T cells in response to human immunodeficiency virus infection. These mRNAs were all Alu rich and unstable sequences. In both reports, however, the relationship of the Alu sequences to their biologic effects was not investigated. Here we have now demonstrated that the 457-bp Alu element is both necessary and sufficient for induction of ST binding activity.

The precise mechanism of this induction remains unknown. Several models, based on current knowledge of the biology of Alu sequences can be proposed. Alu sequences are found in ~10% of mRNAs, and are usually located within the untranslated regions of these RNA polymerase II transcripts (7). Alu sequences have been shown to act in cis, as both transcriptional or post-transcriptional regulatory elements, through RNA-RNA interactions (see Ref. 1, 2, [illegible]). [illegible] are ~90% homologous to the 7SL RNA. Because of this homology, Alu sequences in mRNAs have the potential to modulate gene expression through interactions with cellular proteins of the signal recognition particle as well as other cellular proteins (8, 16). In the present study, Alu sequences in both orientations can induce the ST binding phenotype, and thus may be working through a *trans*-acting mechanism. One possible mechanism for these results is that the transfected Alu sequence is exerting its effect by titrating specific cellular inhibitors that normally regulate transcription or translation (15). Whether the mechanism involves RNA-RNA interactions, or RNA-protein interactions, is presently unknown.

In summary, we have cloned an 1800-bp cDNA that confers ST binding activity in transfected cells, and have discovered that a 457-bp Alu sequence within this cDNA is sufficient to induce expression of a unique cellular receptor. These results suggest that this Alu sequence is part of an expressed mRNA in human tissue, and that it may work through a novel signaling mechanism. We propose that this sequence may belong to a newly emerging class of mRNAs that are untranslated and may play a regulatory role (46) in eukaryotic cell biology.

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2. [illegible]

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EXHIBIT 5

Gut 1994; 35: 1250–1257

Distribution of membrane bound guanylyl cyclases in human intestine

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Abstract

The quantification and distinction of particulate guanylyl cyclases in the human intestine were considered by an enzymatic approach, which comprised the signal transduction from receptor binding to cGMP formation, and, in addition, by showing the expression of an intracellular portion of these transmembrane proteins. Basal guanylyl cyclase (GC) activities were 50 to 80 pmol cGMP formation/min/mg protein and were stimulated up to twofold by heat stable enterotoxin, but were not significantly influenced by atrial natriuretic factor. Enzymatic analysis of colonoscopic specimens pointed to the prevalence of guanylyl cyclase C in the terminal ileum and in the large bowel including colon ascendens, colon descendens, sigmoid, and rectum. The availability of sequence information on human guanylyl cyclases permitted the development of a polymerase chain reaction approach for distinguishing the expression of GC-A and GC-C in human tissue samples. The expression levels of particulate guanylyl cyclases found by polymerase chain reaction in surgical biopsy specimens confirmed the enzymatic data, in that substantial expression of GC-C was found not only in the small intestine but also in the large bowel. According to the restriction mapping of amplificates, GC-C prevailed over GC-A throughout the human intestine, particularly in the mucosal layers.

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Travellers' diarrhoea is one well known example of acute bacterial disease, which reportedly accounts for up to 50% of infant death in developing countries.[1,2] The heat stable enterotoxins produced by several enterobacterial species cause diarrhoeal disease by increasing the cGMP concentrations in intestinal tissue.[3,4] The increase of intracellular cGMP elicited by heat stable enterotoxins decreases water and sodium absorption and enhances chloride secretion by, as yet, not fully understood mechanisms entailing an intestinal isoform of cGMP dependent protein kinase.[1,5] The second messenger cGMP is produced by soluble and membrane bound enzymes.[6–8] The particulate guanylyl cyclases (GC), for example, GC-A and GC-B, are activated in response either to natriuretic peptides, or, in the case of GC-C, to heat stable enterotoxin (ST_a).

We have recently isolated a cDNA encoding a guanylyl cyclase coupled enterotoxin receptor from the human colonic cell line T84.[9] Sequence analysis of this cDNA clone showed the domain division also seen in previously known particulate guanylyl cyclases with comparatively high homologies in the intracellular regions (Fig 1).

While little is known about the distribution of particulate guanylyl cyclases in the intestine of human adults, it has been reported that the increased susceptibility of newborns to diarrhoeal disease parallels the age dependency of GC-C activity[10] and ST_a receptor density[11] in intestine specimens from children. This study was designed to clarify the distribution of particulate guanylyl cyclases, especially GC-A and GC-C, in the intestine of adults. As current textbooks identify the small intestine as the main site for the origin of heat stable enterotoxin induced diarrhoea, we were interested in the occurrence of GC-C also in the colon in comparison with the terminal ileum. The recent discovery of guanylin, a peptide with a high degree of sequence homology with heat stable enterotoxins and stimulatory effects on GC-C,[12] together with the fact that this peptide was purified from the intestine, suggests that guanylin is possibly a physiological regulator of intestinal function and made us investigate the distribution in the intestine of its receptor. The possible presence of GC-A in intestinal mucosa

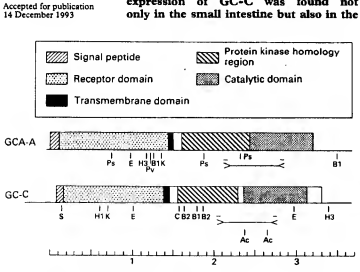

Figure 1: Physical maps of human GC-A and GC-C cDNA. The functional domains postulated according to sequence homologies were assigned to the published cDNA sequences for human GC-A[13] and GC-C.[9] The positions of the binding regions of the common primer pair and the resulting amplification products of about 640 bp are shown below the maps. Additionally some prominent restriction sites are shown and abbreviated as: Ac: AccI, B1: BamH1, B2: BglII, C: ClaI, E: EcoRI, H1: HpaI, H3: HindIII, K: KpnI, Pv: PstI, Pv: PvuI, S: SmaI. AccI sites are shown on the polymerase chain reaction products only.

and the direct participation of atrial natriuretic factor in the origin of diarrhoea has repeatedly been claimed[13-15] or challenged.[16-18] To clarify this point, we examined the occurrence of atrial natriuretic factor stimulated guanylyl cyclase activities and the expression levels of the known GC-A gene in human tissue specimens representing different regions and layers of the intestine.

The aim of this study was therefore to complement and extend current knowledge of the distribution of particulate guanylyl cyclases along the intestine of human adults. Accordingly, the occurrence and frequency of two guanylyl cyclase isoforms, namely GC-A and GC-C, was investigated in the same study. While most previous histochemical studies on particulate guanylyl cyclases used rat tissue, our experiments were conducted on specimens obtained from human adults, an age group that previously has not been examined in this context. For this purpose, biopsy specimens obtained during routine colonoscopies were used for guanylyl cyclase activity assays. To assess clearly the guanylyl cyclase expression pattern in mucosal specimens, contamination of this epithelial layer by blood vessels and smooth muscle from adjacent tissue layers was carefully avoided. Finally, the enzymatic findings were consistently confirmed at the level of gene expression. Guanylyl cyclase basal activities in colonoscopic specimens were monitored and differentially stimulated by addition of either atrial natriuretic factor or heat stable enterotoxin. The molecular expression of particulate guanylyl cyclase genes was investigated by polymerase chain reaction amplification of tissue derived cDNA with subsequent restriction mapping.

Methods

CHEMICALS AND MOLECULAR BIOLOGY REAGENTS

For stimulation of GC-A, rat atrial natriuretic factor (99–126) purchased from the Institut Frappier, Canada, was used. *Escherichia coli* heat stable enterotoxin (ST_a) was obtained from Sigma, St Louis, MO, USA. The Bradford reagent used for protein determinations was obtained from BioRad, Munich, Germany. All other chemicals were of analytical grade and mainly obtained from Sigma.

Magnetic microspheres with covalently attached dT_{25} residues for the purification of mRNA were obtained from Dynal, Oslo, Norway. Custom synthesis of oligonucleotides was performed by MWG, Ebersberg, Germany. Reverse transcriptase from Moloney murine leukaemia virus, Taq-DNA polymerase, and restriction enzymes were purchased from Pharmacia, Freiburg, Germany, Perkin-Elmer, Uberlingen, Germany, and Boehringer Mannheim, Germany, respectively.

TISSUE SPECIMENS

Tissue specimens of about 50 mg each representing different sections of the lower intestine were obtained from four patients between 54 and 62 years of age, who had surgery because of intestinal tumours. Unaffected tissue surrounding the tumours was used. In addition to the mucosal cell layer, which was scraped off before freezing in liquid nitrogen, samples were also collected from the corresponding submucosal muscular layer. The enzymatic studies were based on specimens obtained during colonoscopy from various patients in the age range from 20 to 70 years. Average values of several independent experiments are reported and error bars represent standard errors of the mean.

MEASUREMENT OF GUANYLYL CYCLASE ACTIVITY

Sample preparation for guanylyl cyclase activity assays entailed cell disruption and isolation of membranous cell components. Three colonoscopy samples per membrane preparation were taken up to 500 µl of 20 mM hydroxyethylpiperazine-ethanesulphonic acid (HEPES) buffer pH 7·4, containing 5 mM EDTA, and homogenised with a Potter-Elvehjem homogeniser. Larger surgical samples were suspended in five times their wet weight of the same buffer. Homogenised tissue suspensions were sonicated for 10 seconds and cell debris was removed by low speed centrifugation. Soluble and particulate cell components were separated by 30 minutes of centrifugation at 18 000×*g*. The sediment was resuspended in the original volume of 20 mM HEPES buffer containing 0·5 mM EDTA. Aliquots were frozen in liquid nitrogen and were stored at −70°C. Protein concentrations were measured photometrically at 595 nm according to Bradford.[19]

Guanylyl cyclase activity was determined essentially as described previously.[20] Briefly, 40 µl of reaction buffer were added to 20 µl of appropriately diluted sample for a preincubation at 37°C for three minutes. The guanylyl cyclase reactions were started by the addition, in a volume of 20 µl, of substrate, and stopped after a defined reaction time by addition of 1 ml of a boiling solution of 30 mM EDTA. The final reaction mixture contained 1 mM 1-methyl-3-isobutyl-xanthine, 5 mM creatine phosphate, 500 µg/ml creatine phosphokinase, 0·1 mM amiloride, 1 mM GTP, and 3 mM $MnCl_2$ in 50 mM triethanolamine/HCl buffer, pH 7·4. For differential stimulation of GC-A or GC-C, either 10^{-6} M of atrial natriuretic factor or 10^{-7} M of ST_a were included in the reaction mixture. The amounts of cGMP formed during an appropriate incubation time of usually 30 minutes at 37°C were determined by radioimmunoassay as described previously.[21] Routinely, the membrane preparations from colonoscopies were prediluted for the guanylyl cyclase assay by a factor of 10, and those from surgical biopsies by a factor of three, to yield protein concentrations in the range of 2·5 to 4 mg/ml. Bovine adrenal cortex and porcine ileum served as reference tissues containing atrial natriuretic factor or ST_a stimulatable guanylyl cyclases, respectively,

and were used for assessing the methods of sample preparation and activity assay. Replacing magnesium for manganese in the guanylyl cyclase assay resulted in the high sensitivity for basal activities required for measurements in colonoscopic specimens. The membrane preparations were devoid of soluble guanylyl cyclase because the basal activities in the specimens were not stimulated by 100 μM sodium nitroprusside.

cDNA PREPARATION

Tissue specimens of about 50 mg were ground under liquid nitrogen and lysed in 5 M guanidinium isothiocyanate, 50 mM TRIS-HCl, pH 7·5, 10 mM EDTA, 5% β-mercaptoethanol. Preparation of total RNA was largely according to Chirgwin *et al*,[22] and included the addition of sarkosyl to a final concentration of 2% and heating at 65°C for two minutes. After isopropanol precipitation poly-A^+ RNA was purified using oligo-dT residues coupled to magnetic beads.[23] cDNA was obtained by reverse transcription using random hexamers as primers and Moloney murine leukaemia virus reverse transcriptase at 37°C for one hour in 45 mM TRIS-HCl, pH 8·3, 68 mM KCl, 15 mM dithiothreitol, 9 mM $MgCl_2$, 0·08 mg/ml bovine serum albumin and 1·8 mM of each deoxynucleotide-triphosphate.[24]

POLYMERASE CHAIN REACTION

For the polymerase chain reaction amplification of a stretch of cDNA common to the three known human particulate guanylyl cyclases[9 25 26] a primer pair flanking a 600 bp section of the putative intracellular domain was designed and synthesised chemically. The oligonucleotide sequences were 5′ TG TAC AGC TA/TT GGI ATC ATC 3′ for the upstream primer and 5′ C CAC CAT GTA A/CGC ATC ACC G 3′ for the downstream primer, respectively.

The criteria applied for the design of a primer pair for performing polymerase chain reactions included the specificity for guanylyl cyclases compared with adenylyl cyclases, equal matching to all three known particulate guanyly cyclases, appropriate distance from one another, and minimised secondary structure of both oligonucleotides. The selected primers define an amplificate of 640 bp, which bears different restriction site markers for identifying the three known types of human particulate guanylyl cyclases. The upstream primer confers specificity for particulate guanylyl cyclases and does not match to the known sequences of soluble guanylyl cyclase cDNAs.

The cDNAs encoding human GC-C and rat GC-A were used as test templates for adjusting optimal amplification conditions. Taq-DNA polymerase was used at 1·25 U/50 μl. Unless stated otherwise, the amplification mix contained 10 mM TRIS-HCl, pH 8·3, 50 mM KCl, 1·25 mM $MgCl_2$, 5% glycerol, 200 μM each of the four deoxynucleotidetriphosphates, and 100 nM of each primer. Amplifications were run for 35 cycles consisting for one minute each of denaturation at 94°C, of primer annealing at 56°C, and of DNA synthesis at 72°C. To exclude artefacts in the cDNA amplification three kinds of control polymerase chain reactions were run. Firstly, the addition of template was omitted; secondly, the cDNA template was produced in a mock reverse transcription reaction without tissue RNA; thirdly, to exclude the possibility of DNA contamination in the poly-A^+ RNA preparation, Moloney murine leukaemia virus reverse transcriptase was omitted in a mock first strand cDNA synthesis. None of these controls yielded any amplification products.

Results

GUANYLYL CYCLASE ACTIVITIES IN HUMAN INTESTINE

Membrane preparations from colonoscopies contained 2·5 to 4·0 mg protein per ml and exhibited a linear correlation between the amount of sample and the corresponding guanylyl cyclase activity at a range of total protein concentrations from 50 μg/ml to 1 mg/ml (not shown). The guanylyl cyclase activities of colonoscopic or surgical biopsy specimens in the described assay system behaved in a linear fashion for at least 2·5 hours (not shown).

Figure 2 shows the dose response curves for atrial natriuretic factor and ST_a. These were obtained from membrane preparations derived from colon samples after 30 minutes of incubation in the activity assay. While ST_a provoked a clear rise in cGMP formation, atrial natriuretic factor failed to stimulate the basal guanylyl cyclase activity of this tissue. Similar results were obtained from surgical colon biopsy specimens even after prolonged incubation (not shown). With porcine ileum as the source for ST_a stimulatable guanylyl cyclase a similar concentration response curve as with the biopsy specimens under

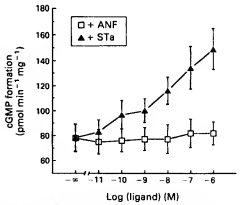

Figure 2: Stimulation of intestinal particulate guanylyl cyclase activities by heat stable enterotoxin and atrial natriuretic factor. Membranous cell components were isolated from colonoscopic specimens taken from the colon descendens. Their specific guanylyl cyclase activities with and without addition of increasing amounts of the ligands atrial natriuretic factor and ST_a were determined from triplicate measurements as described in methods section.

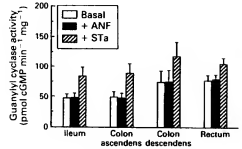

Figure 3: Activities of particulate guanylyl cyclases in different regions of human intestine. The membrane bound enzymes were prepared from colonoscopic specimens taken from the terminal ileum (n=9), colon ascendens (n=9), colon descendens (n=7), and rectum (n=7). Basal as well as atrial natriuretic factor and ST_a stimulated guanylyl cyclase activities were determined after one hour of incubation at the described assay conditions. The particulate guanylyl cyclase activities measured in the colonoscopic specimens were averaged for each of the four different intestine regions.

examination was obtained (not shown). In contrast, GC-A contained in the membrane fraction of bovine adreanal cortex started to respond to atrial natriuretic factor at a ligand concentration of about 1 nM and attained the saturation level of stimulation at about 100 nM atrial natriuretic factor (not shown).

To determine the distribution of particulate guanylyl cyclases in the lower intestine of adult humans, 32 specimens belonging to four different intestine regions were examined enzymatically. Membrane preparations derived from colonoscopic specimens taken from terminal ileum, colon ascendens, colon descendens, and rectum were subjected to guanylyl cyclase activity assays using differential stimulation. Figure 3 shows the basal guanylyl cyclase activities and stimulation by atrial natriuretic factor and ST_a.

The basal activities in terminal ileum and colon ascendens were about 50 pmol cGMP formation/mg protein/min. In colon descendens and rectum the basal activities were around 75 pmol cGMP formation/mg protein/min. Atrial natriuretic factor did not show any stimulatory effect on the guanylyl cyclase activities in any of the tested intestine samples.

After stimulation by 10^{-7} M ST_a, the guanylyl cyclase activities in membrane preparations from terminal ileum and colon ascendens were increased maximally 1·8-fold compared with the basal activities. In colon descendens and rectum, a somewhat lower stimulation of guanylyl cyclase activity by ST_a was seen, namely 1·6-fold or 1·3-fold increases, respectively. The absolute difference between basal and ST_a stimulated specific guanylyl cyclase activities, however, was enhanced from 35 to 45 pmol cGMP formation per min and mg membrane protein from terminal ileum to colon descendens and, with 30 pmol, was slightly lower only in the rectum.

Essentially the same distribution patterns of particulate guanylyl cyclase activities were obtained when specimens representing all four intestinal regions were collected from the same subjects and evaluated for each patient separately (not shown).

COMPARISON OF GUANYLYL CYCLASE ACTIVITIES IN MUCOSAL AND SUBMUCOSAL CELL LAYERS

Surgically obtained tissue samples from colon descendens were used to show the horizontal distribution of particulate guanylyl cyclase activities in the mucosal and submucosal layers of a given intestine section. In Figure 4 the time courses of guanylyl cyclase activities with or without atrial natriuretic factor or ST_a stimulation in mucosal and submucosal membrane preparations are compared. Surprisingly, the basal activities in the submucosal sample surpassed those in the epithelial layer. On the other hand, the stimulation by ST_a after 30 minutes of incubation at 37°C was 1·5-fold in mucosa and only 1·1-fold in submucosa, while atrial natriuretic factor stimulation was negligible in both tissue layers.

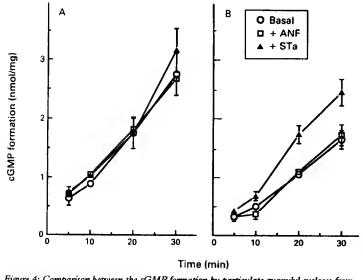

Figure 4: Comparison between the cGMP formation by particulate guanylyl cyclases from mucosal and submucosal cell layers. Tissue samples from the colon descendens were assayed for cGMP formation with or without stimulation by atrial natriuretic factor or ST_a at several time intervals. Specimens obtained after surgery represent submucosal tissue layers (A) and mucosa (B). The time curves show basal activities and guanylyl cyclase activities stimulated by 10^{-6} M atrial natriuretic factor or 10^{-7} M ST_a and obtained from triplicate assays.

AMPLIFICATIONS USING A NEWLY DESIGNED PRIMER PAIR

By sequence comparison we identified short regions common to human GC-A, GC-B_2 and GC-C in the intracellular portions of these membrane proteins and used these to design a primer pair specific for all known particulate guanylyl cyclase cDNAs (Fig 1).

Before using the respective polymerase chain reaction primers for cDNA amplification from intestine samples, a number of control experiments was performed to assure the specificity and sensitivity of the method. For adjusting optimal amplification conditions, recombinant plasmids containing cDNA encoding particulate guanylyl cyclases were

used as test templates in polymerase chain reaction. From human GC-C and rat GC-A cDNA templates, an amplificate of the correct size was obtained. Figure 5 shows that the specificity of amplification could be confirmed by restriction analysis of the polymerase chain reaction products, which exhibited the cleavage patterns expected from the known DNA sequences. The distinction between GC-C and GC-A by restriction mapping also worked, when mixtures of GC-C and GC-A templates were used (not shown). This showed that there was no bias for the amplification of either particulate guanylyl cyclase type. A rough quantification of the message for different particulate guanylyl cyclase types after 35 polymerase chain reaction cycles also was possible, as different template dilutions yielded different signal strengths and because the ratio of template mixtures was reflected in the portions of AccI and PstI cleavable amplificate. To assess the sensitivity of the polymerase chain reaction, recombinant phage lysates with known titres were used. Phage particles containing cloned particulate guanylyl cyclase cDNA were lysed by three freeze-thaw cycles. From as little as 100 plaque forming units of recombinant phage a discernible signal was obtained (not shown). Phage lysates representing cDNA libraries from rat jejunum or the human colonic cell line T84 also yielded amplificates of the size spanned by the particulate guanylyl cyclase primers after 35 cycles of amplification. By restriction analysis, the polymerase chain reaction products amplified from about 10^6 independent clones of these libraries could be assigned to GC-C (not shown).

AMPLIFICATION FROM INTESTINE SPECIMENS

mRNA was purified from about 50 mg of tissue and reversely transcribed. Portions of resulting cDNA were subjected to polymerase chain reaction and the amounts of amplified DNA were estimated after gel electrophoresis. Using the cDNA derived from about 5 mg of tissue, sufficient DNA for a couple of restriction digests was amplified. A typical cDNA amplification experiment from the selected tissue specimens resulted in the electrophoretic banding patterns depicted in those gel lanes that contain uncleaved polymerase chain reaction product (Fig 6).

Whereas the submucosal samples yielded unambiguous DNA fragments of the expected size, additional bands developed in mucosal samples. The sizes of the background bands were smaller than the expected amplification product and common in all mucosal samples. The mucosal caecum sample contained an additional 700 bp band, which, however, was split by AccI into the 300 bp fragments typical for GC-C. The approximate sizes of the smaller fragments found besides the expected amplification product of 635 bp were 350 bp, 450 bp, and 500 bp, respectively. The contrasting electrophoretic appearance after cDNA amplification implies a profound difference between the expression of particulate guanylyl cyclases in mucosa and submucosa.

Because of the comparatively low cycle number and according to the control experiments the amounts of amplificate permit conclusions about the expression levels of particulate guanylyl cyclases in the tissue specimens. A minimum of particulate guanylyl cyclase expression was seen in the caecum. The highest amounts of amplificates from the submucosa preparations were found in the sigmoid and rectum regions. In the mucosal cell layers the highest expression levels were seen in ileum and sigmoid. The content of mRNA encoding particulate guanylyl cyclase in all intestinal specimens by far surpassed that of a control tissue, namely vena saphena magna.

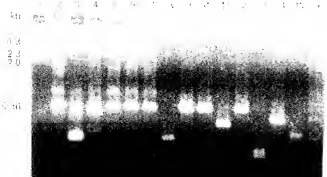

Figure 5: Restriction analysis of amplified particulate guanylyl cyclase cDNA. Plasmid DNA containing human GC-C (lanes 2 to 9) or rat GC-A cDNA (lanes 10 to 16) as inserts was used as the template in polymerase chain reactions under the conditions described. The amplificates were digested with a set of restriction enzymes selected by sequence comparison. Mapping the 640 bp amplificates with this range of restriction endonucleases permitted the two guanylyl cyclase forms to be distinguished from each other. Lanes 1 and 16 contain size markers, while uncleaved amplification product was separated on lanes 2 and 9. The polymerase chain reaction products were digested by the following restriction enzymes: AccI (lanes 3 and 10), AvaII (4, 11), BanII (5, 12), HaeIII (6, 13), PstI (7, 14), and Sau3AI (8, 15) and yielded the predicted fragment sizes.

DISTINCTION OF GUANYLYL CYCLASE FORMS IN AMPLIFIED CDNA

The ratio of different guanylyl cyclase forms in the amplificates was assessed by restriction digests with AccI, which specifically cleaves GC-C amplificates, and PstI, which has recognition sites only on amplificates from GC-A (Fig 1). The polymerase chain reaction products obtained from GC-B cDNA can be discriminated from GC-A and GC-C amplificates by digestion with Sau3AI, which shortens the GC-B amplificate by only 15 bp and BanII which results in 200 bp and 460 bp GC-B fragments. As shown in lanes 2 to 4 of Figure 6 (B), GC-B amplificates also were conspicuous in the present expression assay using the restriction enzymes used to distinguish GC-A and GC-C. Neither the digestion of selection amplificates with Sau3AI and BanII, nor characterisation of the available polymerase chain reaction products by AccI and PstI cleavage yielded any evidence for GC-B expression.

According to restriction analysis, amplificates from submucosa consisted of GC-A

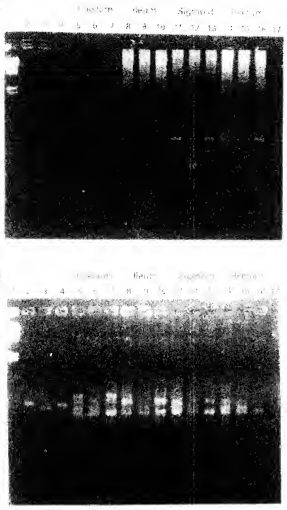

Figure 6: Determination of the prevailing particulate guanylyl cyclase forms in human intestine biopsy specimens cDNA corresponding to 5 mg of tissue was used as a template in the polymerase chain reaction samples, of which one fifth per lane was separated by gel electrophoresis. Amplification products from submucosal (A) and mucosal (B) cDNA represent the intestine regions ileum, caecum, sigmoid, and rectum. The polymerase chain reaction products were electrophorised without cleavage (lanes 2, 5, 8, 11, 15) or after digestion with AccI (lanes 3, 6, 9, 12, 15) or PstI (lanes 4, 7, 10, 13, 16). (A) Additionally shows the restriction mapping of amplificates from blood vessel cDNA, while an amplificate isolated from an adrenal cortex cDNA library and preliminarily classified as GC-B was used in lanes 2 to 4 of (B). As size standards and as a reference for comparing DNA amounts, 500 ng of phage Lambda DNA digested with Hind III (lane 1) and polymerase chain reaction products of known size (lane 17) were included.

and GC-C sequences. Especially in the sigmoid region, GC-C clearly dominated the particulate guanylyl cyclase composition of the submucosa, but the share of GC-C found in submucosal biopsy specimens may result from attached mucosal material. The weak GC-A contribution in submucosal specimens may be attributed to blood vessels and smooth muscle.

Analysis of mucosal amplificates showed the prevalence of GC-C in all examined regions of the intestine, as the specific 640 bp band disappeared after AccI digestion and gave rise to bands corresponding to a size of about 300 bp. Despite the enormous sensitivity of detection provided by the polymerase chain reaction method, there is no indication of GC-A expression in intestinal mucosa as after cleavage by PstI the signal strengths of 640 bp bands fail to decrease and the amounts of 500 bp fragments are not augmented (Fig 6 (B)).

Discussion

A two pronged approach was used to describe the distribution of particulate guanylyl cyclases in human colon. Our results show that GC-C occurs in the large bowel in considerable amounts, with increasing concentrations from the terminal ileum to rectum. No evidence for the existence of GC-A in the mucosa of the lower intestine was detected. As a third interesting finding, we saw comparatively high levels of particulate guanylyl cyclase activity and message in the submucosal cell layers. With regard to the GC-C activities in the lower intestine, our data on colonoscopies for human adults are in good agreement with the values recently measured in the rat by Mezoff *et al*,[27] if the lower assay temperature of 32°C used by these authors is taken into account. The presence of GC-C in the lower intestine is not surprising, as the corresponding cDNA had been isolated from a colonic cell line,[9] an acknowledged source of ST_a – receptor.[12] Our enzymatic results on the distribution of GC-A and GC-C in human tissue specimens are also in good agreement with recent data by Vaandrager *et al* [28] using a similar approach of differential stimulation by atrial natriuretic factor and ST_a with freshly isolated rat intestinal crypt and villus cells, stripped mucosa of proximal rat colon, and various colon carcinoma and undifferentiated small intestinal cell lines.

Above all, in the rectum, but to a lesser extent also in the other intestinal sections, a high proportion of particulate guanylyl cyclase activity was seen, which was neither stimulated by atrial natriuretic factor or by ST_a. These high levels of non-stimulatable basal activities can be explained at least partly by the assay method. The choice of manganese as the divalent cation resulted in higher basal activities and less stimulation. This effect was most pronounced with the high basal guanylyl cyclase activities found in the rectum. The detection of low basal activities was a prerequisite for performing measurements on minute colonoscopic samples, but accounts, to some extent, for the weak stimulation. As GC-C clearly prevailed in the colonoscopic samples, receptor destabilisation by the ligand ST_a[29] offers an additional explanation for the high basal guanylyl cyclase activities.

By the described polymerase chain reaction approach, we have shown expression of particulate guanylyl cyclases in human intestine samples and additionally could determine the prevalence of one of the three forms. The primer binding regions as well as the cleavage patterns of the selected restriction enzymes turned out to be sufficiently conserved to make our approach applicable to several mammalian species. Therefore, in view of the small amounts of tissue required, the present polymerase chain reaction method provides an attractive alternative to a northern blot procedure. It is one of the strengths of the

present expression assay, that it can show the distinction between particulate guanylyl cyclase types by restriction mapping, as hybridisation procedures are not suitable for this purpose, when cDNA encoding the highly conserved intracellular portions of the transmembrane receptors are involved.

On the gene expression level, hybridisation of a GC-A cDNA probe to total RNA from human intestine was found.[25] As this large hybridisation probe contained the intracellular protein kinase and catalytic domains that are homologous in all known particulate guanylyl cyclases, the northern hybridisation signals from intestinal tissue can be interpreted as GC-C expression. In rat jejunum, ileum, and colon GC-C expression was also shown by northern hybridisation probing for the message encoding the extracellular receptor domain.[30] Extracting the information about the adult stage from this study,[30] it becomes evident that the GC-C expression level in the large bowel is about equal to that in the small intestine. The hybridisation data from the rat thus coincide with our finding by polymerase chain reaction for adult humans.

Enzyme activity and gene expression measurements support one another in two crucial points: the lower human intestine contains considerable concentrations of GC-C, while GC-A seems to be virtually absent from the mucosal layers. While ST_a stimulated GC-C activity was found in the colon at values even surpassing those in the ileum, no substantial atrial natriuretic factor stimulation of guanylyl cyclase activity was found in any of the biopsy specimens. In the mucosal layers even the extremely sensitive polymerase chain reaction assay failed to detect any expression of GC-A. This is in contrast with previous findings on the distribution[13 14] and physiological role[15] of GC-A in the intestine, but supports other previous data.[16 17] In rat intestinal mucosa, a twofold increase of particulate guanylyl cyclase after atrial natriuretic factor application was reported,[13] but these data have been contradicted by Tremblay *et al*,[16] who found no activation of guanylyl cyclase in isolated intestinal epithelial cells after incubation with atrial natriuretic factor. Reports on the location of GC-A and also the functional effect of atrial natriuretic factor in the intestine are also contradictory. Thus, a decrease of intestinal water absorption in response to atrial natriuretic factor application was found in rats by Moriarty *et al*,[15] whereas other researchers[16–18] did not find any atrial natriuretic factor induced changes of the intestinal water balance in human subjects. There are at least three ways to explain these discrepancies. Firstly, a difference in atrial natriuretic factor receptor distribution may exist between species, in the sense that the situation in human intestine differs from that in other mammals.[31] Secondly, the GC-A activities found by Waldman *et al*[13] might stem from submucosal tissue. Thirdly, the intestinal atrial natriuretic factor binding sites shown by Bianchi *et al*[18] might present atrial natriuretic factor clearance receptor[32] rather than GC-A. The distribution of GC-A and GC-C in the mucosal and submucosal layers of rat intestine has been shown by differential stimulation in situ with subsequent immunodetection of formed cGMP.[28] Our measurements with stripped mucosa and corresponding submucosal tissue specimens confirm these results for different regions of the human lower intestine and additionally establish a congruent pattern of particulate guanylyl cyclase expression.

It is tempting to compare the tissue distributions of GC-C and its endogenous ligand, guanylin.[33 34] The levels of mRNA encoding the guanylin precursor in the gastrointestinal tract of the rat increase strikingly from the oesophagus to the colon.[34] We have shown for the lower intestine of humans that the corresponding receptor shows a similar distribution, although the message of the guanylin precursor might be far less abundant, as shown by a comparison of the frequency of GC-C and guanylin cDNA in a commercially available rat jejunum library (G Krause, unpublished data). As a circulating 10 kDa form of guanylin has been purified from human haemofiltrate,[34] guanylin and GC-C may be regarded as hormone and hormone receptor, with additional roles outside their common main site of synthesis. This report on the functional and molecular expression of particulate guanylyl cyclases in the colon suggests that the large bowel might contribute considerably to the origin of ST_a induced diarrhoea or to the guanylin regulated fluid balance.

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Parts of the results have been presented at the 8th Conference on Second Messengers and Phosphoproteins at Glasgow in August 1992. Some enzymatic data also are contained in Anja Bayerl's MD thesis.

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EXHIBIT 6

Proc. Natl. Acad. Sci. USA
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Cell Biology

Enterochromaffin cells of the digestive system: Cellular source of guanylin, a guanylate cyclase-activating peptide

(gut hormones/entero-endocrine cells/intestine/heat-stable enterotoxin receptor/diarrhea)

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ABSTRACT **Guanylin, a bioactive peptide, has recently been isolated from the intestine; this peptide activates intestinal guanylate cyclase (i.e., guanylate cyclase C) and thus is potentially involved in the regulation of water/electrolyte transport in the gastrointestinal mucosa. As yet, the cells involved in synthesis, storage, or secretion of guanylin have not been identified by immunocytochemistry. We raised antisera against guanylin and investigated the entire gastrointestinal tract of guinea pigs by light and electron microscopical immunocytochemistry. Extracts of various intestinal segments and plasma analyzed on a Western blot revealed a peptide band corresponding to the molecular mass of guanylin. Localization studies in the entire digestive tract showed that guanylin is exclusively confined to enterochromaffin (EC) cells. Remarkably, most EC cells contacted the gut lumen by cell processes that were highly immunoreactive for guanylin. In addition to the well known secretion in an endocrine fashion, EC cells by circumstantial evidence may release guanylin into the gut lumen to activate guanylate cyclase C that is immediately located on the brush border of adjacent enterocytes. The unique localization of guanylin in EC cells may indicate that these cells are involved in the regulation of fluid secretion in the gastrointestinal mucous membrane.**

Heat-stable enterotoxins (STa), small peptides of 18 or 19 amino acids, cause secretory diarrhea when secreted into the intestine by enterotoxigenic strains of *Escherichia coli* (1). A high-affinity receptor for this class of diarrheal enterotoxins has been demonstrated in the intestinal brush border membranes (2, 3). Recently, guanylate cyclase C (GC-C) has been identified as the cell surface receptor for STa (4, 5); it has been proposed that STa merely mimic the actions of an endogenous ligand for GC-C (6, 7). Guanylin, a 15-amino acid peptide, has been isolated from jejunal extracts and identified as endogenous activator of GC-C (8). As described for STa (6, 9), guanylin elicits an increase in cellular cGMP that mediates the increase in chloride secretion and the decrease in water absorption, finally causing secretory diarrhea (8, 10). Human, rat, and mouse cDNAs encoding the precursor of guanylin have been cloned and characterized (10–12). The prohormone consists of 115 amino acids containing at its C terminus the peptide isolated from the intestine (8).

Northern blot analysis of human and rat guanylin mRNA levels in various organs revealed that guanylin is mainly expressed in the intestine (10, 12) with the highest expression levels in the colon (11, 12); by using *in situ* hybridization techniques, guanylin mRNA was found in cells located at the base of small intestinal crypts, namely Paneth cells (10), a cell type that has a tendency to bind nonspecifically nucleic acid probes (13). Moreover, this cell type is restricted almost completely to the small intestine and is not present in the colon of most species (13, 14). On the other hand, high amounts of circulating high molecular mass guanylin were demonstrated in the blood (15), indicating that guanylin may originate primarily from endocrine sources in the gut. Therefore, we raised antisera against the midportion and against the C terminus of the guanylin molecule, characterized them by Western blot analysis, and report here the cellular and subcellular localization of guanylin in the guinea pig gastrointestinal tract.

MATERIALS AND METHODS

Peptide Synthesis. From the published human guanylin sequence (10, 11), the following guanylin peptides were synthesized: guanylin-(34–46), guanylin-(101–115), and [Lys^{100}]guanylin-(101–115). Guanylin-(34–46) was synthesized on a SMPS 350 automated multiple peptide synthesizer (Zinsser, Frankfurt, F.R.G.) using the standard Fmoc protocol (16). Peptide chains were assembled by TBTU/DIPEA/HOBT activation on a Wang resin. The sequence was synthesized as a linear peptide and as an octameric multiple antigenic peptide (for immunization; see ref. 17). Guanylin-(101–115) and [Lys^{100}]guanylin-(101–115) were synthesized manually on Fmoc-Cys(Trt)-Wang resin using double coupling cycles, monitoring by Kaiser test, and end-capping with acetic acid anhydride/pyridine, 2:1 (vol/vol). The cystine bridge between residues 107 and 115 was introduced by oxidation with potassium ferricyanide (III), and the cystine bridge between residues 104 and 112 was introduced by oxidation with iodine. The purity and sequence of the synthesized peptides were checked by reverse-phase HPLC [Vydac (Hesperia, CA) C_{18}], capillary zone electrophoresis (Bifocus 3000, Bio-Rad), mass spectrometry (Sciex API III, Perkin–Elmer), automated Edman degradation (model 473A protein sequencer, Applied Biosystems), and amino acid analysis (Aminoquant 1090L, Hewlett–Packard).

Immunization Procedure. [L-ys^{100}]Guanylin-(101–115) (0.5 mg per rabbit) was conjugated to limpet hemocyanin (Sigma) by using carbodiimide as coupling agent. Guanylin-(34–46) (0.5 mg of multiple antigenic peptide per rabbit) was dissolved in saline (1 mg/ml). Rabbits (New Zealand White, five for each antigen) were immunized subcutaneously with guanylin-(34–46) and [Lys^{100}]guanylin-(101–115) conjugates emulsified in complete Freund's adjuvant at 1:1 (vol/vol). They were given booster injections every 4 weeks and bled 14 days after each booster injection. The titer of the antisera against the corresponding linear peptides was tested in enzyme-linked immunosorbent assay. Out of the antisera raised, the antisera K42 recognizing guanylin-(34–46) and

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Abbreviations: GC-C, guanylate cyclase C; EC, enterochromaffin.

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K605 recognizing guanylin-(101–115) had the highest titers and were used for detailed investigations.

Extraction of Guanylin from Plasma and Tissues. Guinea pigs ($n = 2$) were anesthetized by ether inhalation. Blood (2 ml) was collected into ice-chilled tubes containing K_2EDTA and centrifuged at 2000 × g for 20 min at 4°C. Plasma (1 ml) was diluted 1:1 (vol/vol) with 0.01 M HCl and adjusted to pH 3.0 by concentrated HCl. Tissue specimens from duodenum, jejunum, ileum, and colon were flushed of luminal contents with ice-cold saline. Skeletal muscle was taken as control tissue. All tissues were immediately boiled in 1 M acetic acid for 10 min and homogenized with an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, F.R.G.). The homogenates were centrifuged at 20,000 × g for 20 min at 4°C and the supernatants were filtered through a 0.45-μm (pore size) filter. Plasma and acid tissue extracts were applied to an octadecasilyl (C_{18}) Sep-Pak cartridge (Waters). The column was washed with 0.01 M HCl and material was eluted with 30% (vol/vol) 2-propanol/30% (vol/vol) methanol/0.01 M HCl. The eluted protein fractions were lyophilized and stored at −80°C until use.

Western Blots. For immunoblot analysis, plasma and tissue extracts were incubated for 7 min at 95°C in sample buffer with 4% (wt/vol) SDS (Merck), 50 mM Tris·HCl (pH 8.45), 1 mM EDTA, 3.24 mM dithiothreitol (Roth, Karlsruhe, F.R.G.), 12.5% (wt/vol) glycerol (Merck), and 0.002% bromphenol blue (Merck) (reducing conditions). The samples were separated by tricine–SDS/PAGE in 16.5% gels by the method of Schägger and von Jagow (18). Low molecular mass markers (Boehringer Mannheim) were used for the molecular mass calibration. After electrophoresis, proteins were electroblotted onto hydrophobic polyvinylidene fluoride-based membranes (Pall). To block unspecific binding of antibodies, blot strips were incubated in 5% (wt/vol) skin milk in Tris-buffered saline (TBST) containing 10 mM Tris·HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20. After washing in TBST, the membranes were incubated overnight at 4°C with antisera K42 (diluted 1:1500 in TBST) and K605 (diluted 1:250 in TBST). Immunoreactive proteins were visualized after incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (diluted 1:8000; Sigma) using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as chromogens (Sigma). Proteins remaining in the gels were fixed in 30% methanol/10% acetic acid for 45 min before they were stained with Coomassie brilliant blue in 10% acetic acid/30% methanol for 1–2 h.

Tissues and Tissue Preparation for Immunocytochemistry. Small specimens of the mucosa of all segments of the guinea pig gastrointestinal tract ($n = 10$) were snap-frozen in Freon 22 precooled with liquid nitrogen, freeze-dried, and fixed by vapor-phase paraformaldehyde (19) or diethyl pyrocarbonate (20). For immunoelectron microscopy, small specimens from the same tissues were fixed by immersion in periodate/lysine/paraformaldehyde (PLP) (21) for 18 h without postosmification, rinsed overnight in 0.05 M sodium phosphate-buffered (pH 7.3) 0.15 M NaCl (PBS) and dehydrated in a graded ethanol series. All specimens were embedded in epoxy resin (Araldite).

Antisera for Immunocytochemistry. The following antisera were used to identify cells: both guanylin antisera (K42 and K605), antisera against chromogranin A and lysozyme, and 13 rabbit antisera against the various gastrointestinal hormones—bovine adrenal medulla octacapeptide (BAM-12P), cholecystokinin, gastrin, gastric inhibitory polypeptide, glucagon, histamine, motilin, neurotensin, bovine pancreatic polypeptide, peptide tyrosine tyrosine, secretin, serotonin, and somatostatin. Most of these antisera have been characterized and used in previous investigations (see refs. 22–27).

Immunocytochemical Protocol. *Light microscopy.* Serial semithin sections were cut at 0.5 μm and mounted on microscope slides. After removal of the epoxy resin by sodium methoxide (22, 23), the sections were immunostained by the avidin–biotin–peroxidase complex (ABC) technique (28): serial sections were alternatively incubated with guanylin antisera (diluted 1:2000–4000) and with the antisera for cell identification (diluted 1:1000–32,000) for 24 h at 4°C, followed by incubation with the second antibody, biotin-labeled goat anti-rabbit IgG (Jackson ImmunoResearch) for 30 min diluted 1:200. The sections were then incubated for 30 min with a preformed complex of biotin–peroxidase/streptavidin (Jackson ImmunoResearch), diluted in PBS (final concentrations: biotin-peroxidase, 0.7 μg/ml; streptavidin, 5 μg/ml). The antigen–antibody binding sites were visualized by incubation of the sections in 0.7 mM diaminobenzidine hydrochloride/0.002% H_2O_2 in 0.05 M Tris·HCl (pH 7.6). PBS was used as diluent for the antisera and as rinsing solution.

Electron microscopy. Ultrathin sections were immunostained by both guanylin antisera (diluted 1:1000) using the immunogold technique as described (23). Thereafter, the sections were air-dried, counterstained with uranyl acetate for 10 min, rinsed in H_2O for 2 min, and viewed in an electron microscope (Zeiss EM 9S-2).

Specificity Controls. Method-dependent nonspecificities were excluded by running controls as described (see refs. 22 and 23). Antibody specificities were tested by preadsorption of the antisera with homologous and heterologous antigens (see below) at 6.25–100 μg/ml of antiserum (working dilution). Preadsorption of the antiserum K42 with guanylin-(34–46) at concentrations as low as 6.25 μg/ml completely blocked immunostaining. The same was true for preadsorption of the antiserum K605 with guanylin-(101–115). Preadsorption of the antiserum K42 with guanylin-(101–115), as well as with various antigens (listed below), at concentrations up to 100 μg/ml had no effect on immunostaining. Likewise, immunostaining with antiserum K605 was not affected by preadsorption with guanylin-(34–46) and with various unrelated antigens at concentrations up to 100 μg/ml. The antisera against chromogranin A and serotonin could be blocked by preadsorption with the corresponding antigens (at 6.25 μg/ml) but not with guanylin-(34–46) and guanylin-(101–115) (at 100 μg/ml).

Antigens for Preadsorption Controls. Chromogranins (purified from adrenal medullary extracts) were provided by M. Gratzl (Ulm, F.R.G.). Chromostatin was obtained from D. Aunis (Strasbourg, France). Cholecystokinin was provided by W. Schlegel (Munster, F.R.G.). Bovine pancreatic polypeptide was a gift of R. E. Chance (Indianapolis). Guanylin-(34–46) and guanylin-(101–115) (see above); glucagon, serotonin creatinine sulfate, and histamine hydrochloride (all from Serva); BAM-12P, gastrin, gastric inhibitory polypeptide, limpet hemocyanin, motilin, and peptide tyrosine tyrosine (all from Sigma); insulin (Novo Industries, Bagsvaerd, Denmark); and α-neo-endorphin, dynorphin A, neurotensin, pancreastatin, secretin, somatostatin, and substance P (all from Peninsula Laboratories) were obtained as indicated.

RESULTS

Western Blot Analysis. Both guanylin antibodies (K42 and K605) recognized a major intestinal peptide of 10–12 kDa in all lanes loaded with intestinal tissue extracts (Fig. 1). In addition, intensive labeling of a plasma peptide in the same range (10–12 kDa) was obtained with the antiserum K605 but not with K42. With both antisera, a faintly stained band was also visualized at 17–19 kDa in lanes loaded with intestinal proteins. Any crossreactions with the second goat anti-rabbit antibody were excluded by appropriate controls. Western blot analysis of skeletal muscle extracts (control), processed

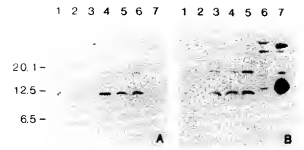

FIG. 1. Western blot of guinea pig tissues and plasma extracts (after tricine–SDS/PAGE electrophoresis) immunostained with antiserum K42 (*A*) and antiserum K605 (*B*). (*A*) Lanes: 1, colon; 2, plasma; 3, skeletal muscle; 4, ileum; 5, jejunum; 6, duodenum; 7, molecular mass markers. (*B*) Lanes: 1, colon; 2, molecular mass markers; 3, duodenum; 4, jejunum; 5, ileum; 6, skeletal muscle; 7, plasma. Note the predominant immunoreactive band of 10–12 kDa obtained with both antisera recognizing different epitopes in the guanylin molecule. Molecular mass markers are indicated (20.1 kDa, a trypsin inhibitor; 12.5 kDa, cytochrome *c*; 6.5 kDa, aprotinin).

on the same gel, showed neither the major immunoreactive band of 10–12 kDa nor the minor band at 17–19 kDa.

Immunocytochemistry. *Light microscopy.* Both guanylin antisera coincidingly immunostained distinct cells located in the epithelium throughout the gastrointestinal tract (Figs. 2 and 3). Within the gastric mucosa, such cells were rarely present; in the upper small intestine, they were numerous; in the lower small intestine and especially in the colon, guanylin-immunoreactive cells occurred rarely. In serial sections, guanylin-immunoreactive cells were also immunostained for chromogranin A, a glycoprotein used as marker for various endocrine cells (24, 29). Hence, guanylin was localized in endocrine cells. Of the various endocrine cell types in the entero-endocrine system (24–26, 30) immunostained by appropriate antisera, exclusively the enterochromaffin (EC) cells (identified by the serotonin antiserum) exhibited immunoreactivity toward both guanylin antisera (Figs. 2 and 3). These cells were located predominantly in the crypt regions but they were also present in the epithelium of the villi of the small intestine. Basically, EC cells of both "closed" and "open" types (26, 30) showed immunoreactivities for guanylin (Fig. 3). However, dependent on the segment investigated, the EC cells were heterogeneous with respect to their guanylin immunoreactivities. In the stomach, EC cells predominantly of the antral pyloric region exhibited immunoreactivity for both guanylin peptides (Fig. 2). In the duodenum, all EC cells detected showed guanylin immunoreactivity. Although many EC cells were present in the remaining intestinal segments, the frequency of guanylin-immunoreactive EC cells continuously decreased from jejunum toward colon, as estimated by comparison of serial sections alternatively immunostained by serotonin and guanylin antisera. Particularly in the colonic mucosa, most EC cells were unreactive toward both guanylin antisera. Beyond such variations in frequency, differences in staining characteristics of the guanylin antisera in the same EC cells were evident. Although in all gastrointestinal segments generally the same EC cells were immunostained by both guanylin antisera, a minority of EC cells displayed immunoreactivity for guanylin-(101–115) but lacked completely or showed faint immunoreactivity for guanylin-(34–46). In no case was immunoreactivity for guanylin found in other epithelial or nonepithelial cells including gastric gland cells and Brunner's gland cells. Paneth cells of the small intestine identified by their lysozyme content (14) were definitely unreactive toward both guanylin antisera.

Electron microscopy. Guanylin-(34–46) and guanylin-(101–115) immunoreactivities were exclusively localized in polymorphous secretory granules typical for EC cells (Fig. 3) that also contain serotonin and chromogranin A (27). The secretory granules were densely and homogeneously labeled by both guanylin antisera.

DISCUSSION

By cDNA analysis, the primary amino acid sequence of guanylin has been elucidated (10–12). Northern blot analyses in various organs showed high-level expression of guanylin mRNA restricted to the intestine (10). Recently, high amounts of guanylin were found in plasma (15), indicating that this peptide may originate mainly from endocrine sources in the intestine. Therefore, the present study was focused on the endocrine system of the gut to determine the cellular and subcellular localization of guanylin. By using two guanylin antisera directed against different epitopes, a predominant immunoreactive peptide in the range of 10–12 kDa was identified by Western blot analyses of extracts of all segments of guinea pig intestine but not of skeletal muscle extracts (negative control). This molecular mass is in accordance with the mass of circulating human guanylin (10.3 kDa; see ref. 15) as well as with the molecular mass deduced for the guanylin prohormone from the cDNA sequence (12.5 kDa; see ref. 10). As described for humans (15), guanylin seems to circulate as a high molecular mass peptide in guinea pig

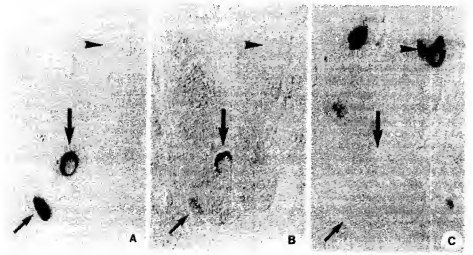

FIG. 2. Three semithin (0.5 μm) serial sections of guinea pig pyloric mucosa immunostained for serotonin (*A*), guanylin (antiserum K42) (*B*), and gastrin (*C*). Guanylin immunoreactivity is present only in EC cells identified by the serotonin antiserum. The EC cells shown contain strong (large arrows) or faint (small arrows) immunoreactivity for guanylin. Gastrin cells are devoid of guanylin immunoreactivity (arrowheads). Interference-contrast microscopy. (×520.)

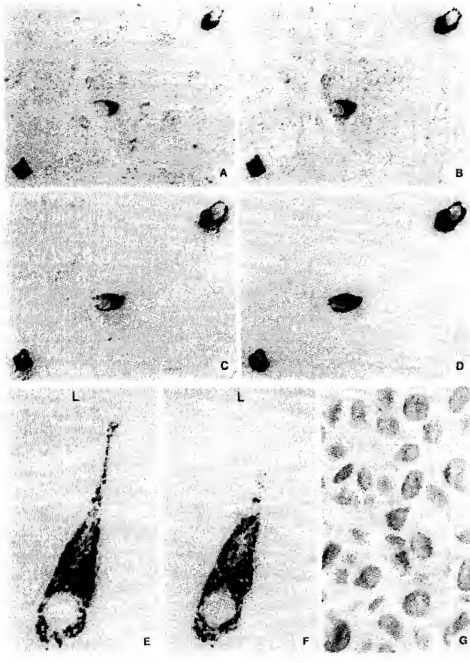

FIG. 3. (*A–D*) Four semithin (0.5 μm) serial sections of the duodenal mucosa immunostained by the guanylin antisera K42 (*A*) and K605 (*B*) and antisera against serotonin (*C*) and chromogranin A (*D*). Three EC cells, identified by the serotonin antiserum, simultaneously exhibit strong immunoreactivities for guanylin-(34–46), guanylin-(101–115), and chromogranin A. (*E* and *F*) Two semithin sections of the jejunal mucosa immunostained for serotonin (*E*) and guanylin-(101–115) (antiserum K605) (*F*). An EC cell (jejunal mucosa) contains strong serotonin and guanylin immunoreactivities predominantly localized at the supranuclear site. This EC cell contacts the lumen (L) through a slender cellular process. (*G*) Subcellular localization of guanylin (antiserum K605) in a duodenal EC cell by the immunogold technique. Guanylin immunoreactivity is confined to polymorphous secretory granules typical for EC cells. *A–F*, interference-contrast micrographs; *G*, electron micrograph. (*A–D*, ×495; *E* and *F*, ×1270; *G*, ×20,025.)

blood, since immunoreactivity of a 10- to 12-kDa plasma peptide was detected with the antibody K605 (directed against the C terminus of guanylin). In addition, both antisera did recognize with lower affinity another intestinal protein of higher molecular mass (17–19 kDa).

Immunocytochemical investigations revealed that among the various entero-endocrine cell types (24–26, 30) identified here, only the EC cells contain guanylin immunoreactivity. The EC cell, regularly containing serotonin (31, 32), is the most abundant endocrine cell type in the gastrointestinal tract and is common to all segments of the gut (30). The coinciding staining by both guanylin antisera not only in a defined endocrine cell population but also in mostly the same cells unequivocally indicates that guanylin is specifically localized in EC cells of the gut. This is further substantiated by the subcellular localization of guanylin, which is exclusively confined to EC-cell secretory granules—i.e., a compartment where other secretory products of these cells (serotonin and chromogranin A) are also present (27). The present findings, moreover, showed that depending on the segment investigated, the EC cells are heterogeneous with respect to their guanylin immunoreactivities. Although by far most EC cells displayed guanylin immunoreactivity in the upper small intestine, many EC cells were unreactive toward both guanylin antisera in the lower parts of the small intestine and, especially, in the large intestine. In accordance with these findings, Western blot analyses with both guanylin antisera revealed faint guanylin immunoreactive band in the large intestine compared to those in the small intestine. On the other hand, increasing levels of guanylin mRNA expression have been demonstrated from duodenum toward colon (11, 12). Possibly, EC cells in the lower parts of the intestine, in contrast to the upper segments, are characterized by high synthesis and secretion turnover for guanylin resulting in

minimal content or in lack of guanylin storage in these cells thus escaping detection by the respective antisera at dilutions used. Remarkably, in the gastric mucosa a small number of EC cells exhibited immunoreactivity for guanylin, although recent studies showed lack of guanylin mRNA expression in the stomach (11, 12), possibly due to minimal amounts of guanylin mRNA in the stomach falling below the sensitivity of the detection system.

From the fact that both guanylin antisera, recognizing different epitopes in the guanylin molecule, yield immunoreactivities concomitantly localized in most EC cells of all tissues investigated, we assume that these cells contain the entire guanylin molecule. The heterogeneous immunoreactivities for guanylin-(101–115) and guanylin-(34–46) found in a small subpopulation of EC cells may be related to intercellular differences in posttranslational modifications or processing of the guanylin molecule but also to a possible existence of unknown guanylin-related peptide(s) in EC cells, especially in view of the immunoreactivity pattern of the guanylin antisera in intestinal extracts as verified by Western blots.

EC cells as typical members of the "diffuse neuroendocrine system" (33) are scattered in the gastrointestinal epithelium. In this location, closed type and open type EC cells have been identified based on their relationship to the gut lumen (30). EC cells are generally accepted as typical endocrine cells of the gut; release of serotonin, the main EC-cell product, from the gastrointestinal tract into the circulation is well documented (34, 35). However, increasing evidence exists also for an endoluminal release of EC-cell secretory constituents (36–38); such a luminocrine secretory pathway is attributed to open type EC cells that reach the gut lumen by apical cell processes (30, 36). Indeed, release of serotonin into the gut lumen has clearly been demonstrated (38–40). Since guanylin is present in EC-cell secretory granules where serotonin is also localized, EC cells predictably release guanylin not only into the circulation but also into the gut lumen. Secretion of guanylin by EC cells through an endocrine pathway is certainly in line with the strong guanylin immunoreactivity in guinea pig plasma as demonstrated here by Western blot analysis and with the high amounts of guanylin found in human plasma (15). In addition, a luminocrine secretion of guanylin from EC cells is quite conceivable by circumstantial evidence and would allow interaction of this peptide with GC-C immediately localized on the brush border of enterocytes (2, 3) to regulate intestinal fluid secretion. Although the EC cell is the most numerous endocrine cell type in the gastrointestinal tract known for several decades, its true function(s) hitherto remained enigmatic. At least one function of EC cells is apparently related to local regulation of water and electrolyte secretion in the gastrointestinal epithelium through a luminocrine secretory pathway. The luminocrine secretory activity of EC cells is obviously under vagal control (39, 40), and interpreted retrospectively, diarrhea observed after stimulation of EC cells (41, 42) may be attributed to guanylin secreted by these cells into the gut lumen. In this respect, future studies should focus on the regulation of guanylin biosynthesis in EC cells and in tumors derived from them (e.g., carcinoids) of which one predominant and common effect is diarrhea.

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EXHIBIT 7

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Biochemistry

Regulation of cell signaling by the cytoplasmic domains of the heat-stable enterotoxin receptor: Identification of autoinhibitory and activating motifs

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ABSTRACT **Infection with enterotoxigenic *Escherichia coli* is a leading cause of traveler's diarrhea. Many enterotoxigenic *E. coli* strains produce heat-stable enterotoxin (ST), a peptide that binds to the intestinal receptor guanylyl cyclase C known as STaR. The toxin–receptor interaction elevates intracellular cGMP, which then activates apical chloride secretion, resulting in secretory diarrhea. In this report, we examine how the intracellular domains of STaR participate in the propagation and regulation of signaling. We show that STaR exists as an oligomer in both the presence and the absence of toxin. We also demonstrate that deletion of the intracellular kinase-homology domain produces a constitutively active mutant, suggesting that this domain subserves an autoinhibitory function. Finally, we constructed a point mutant within a highly conserved region of the cyclase domain that completely inactivates the catalytic activity of guanylyl cyclase. Cotransfection of this point mutant with wild-type receptor causes a dominant-negative effect on receptor activation. This suggests that interaction of receptor subunits is required for toxin-induced activation and that the cyclase domain is involved in this essential interaction. We propose that the binding of ST to STaR promotes a conformational change across the cell membrane. This removes the inhibitory effects of the kinase-homology domain and promotes an interaction between cyclase domains that leads to receptor activation. The data suggest a paradigm of signal transduction that may also be relevant to other members of the guanylyl cyclase receptor family.**

Infection with enterotoxigenic *Escherichia coli* is a leading cause of traveler's diarrhea (1). Many pathogenic human enterotoxigenic *E. coli* strains secrete ST, a heat-stable small cysteine-rich peptide toxin that binds to the intestinal receptor guanylyl cyclase C known as STaR. The binding of ST to this receptor activates the intracellular guanylyl cyclase domain of the receptor, causing substantial increases in the intracellular cGMP content (2–4). Secretory diarrheal symptoms are believed to result from cross-activation of protein kinase A by cGMP, which in turn stimulates chloride secretion (5–7).

STaR is a member of the guanylyl cyclase receptor family. The intestinal peptide guanylin is the putative endogenous receptor ligand (6, 8–10). Other members of this receptor family include the atrial natriuretic peptide (ANP) receptors (GC-A and GC-B), the retinal-specific guanylyl cyclase, and the sea-urchin sperm guanylyl cyclase (11). Though the receptors regulate diverse physiologic processes through the second messenger cGMP, they have a similar cellular topology. This includes an N-terminal extracellular ligand-binding domain, a single transmembrane domain, a kinase-homology domain, and a C-terminal guanylyl cyclase catalytic domain. The cyclase domain is highly conserved among members of this receptor family. In contrast, the ligand-binding and the kinase-homology domains have limited amino acid sequence homology, consistent with their different functions and ligand specificities.

The molecular events leading to signal transduction by the guanylyl cyclase receptor family are not well understood and have not been previously studied for STaR. In this report, we examine how the intracellular domains of the receptor participate in the propagation and regulation of signaling. We present evidence that oligomeric interactions of STaR occur in both the presence and absence of ligand and that deletion of the kinase-homology domain constitutively activates the receptor. We describe a point mutation within the cyclase domain that exerts a dominant-negative effect. Interaction of receptor subunits within the cyclase domain is needed for activation, and the kinase-homology domain may serve to inhibit interaction of the cyclase domains in the absence of ligand.

METHODS

Plasmid Constructions. The 3.4-kb cDNA for the human ST receptor (wt-STaR) was subcloned from Bluescript SK$^-$ (3) into the *Xho* I and *Pst* I sites of pMT28S (12), a simian virus 40-based expression vector. Epitope-tagged receptors (13) were prepared by inserting the influenza hemagglutinin (HA; YPYDVPDYA) or FLAG (DYKDDDDK) epitope immediately C-terminal to the putative signal sequence cleavage site using PCR (14). To facilitate subcloning, a silent mutation in wt-STaR was introduced to provide the unique restriction site *Cla* I at nucleotide 1521. Mutant constructs kin$^-$, cyc$^-$, and D834A were also constructed with PCR, using HA or FLAG epitope-tagged wt-STaR as the template. kin$^-$ lacks the kinase-homology domain, from amino acid 441 to amino acid 736. For mutant D834A, a single point mutation was made in the cyclase domain of FLAG-tagged wt-STaR, substituting an alanine for aspartic acid at residue 834. cyc$^-$ encodes a receptor truncated at amino acid 682 and thus lacks the entire cyclase domain. ecd was prepared by *Xba* I and *Bgl* II digestion (nucleotide 1597), followed by blunt ending and religation. DNA fragments that were obtained by PCR amplification were verified by dideoxy sequencing. Schematic representations of these constructs are depicted in Table 1.

Cell Culture and Transfections. COS cells were maintained in DMEM with 10% (vol/vol) heat-inactivated fetal calf serum and penicillin (100 units/ml)/streptomycin (100 μg/ml). Cells

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Abbreviations: ST, heat-stable enterotoxin; STaR, ST receptor; mAb, monoclonal antibody; HA, influenza hemagglutinin; ANP, atrial natriuretic peptide.

†X.L.R. and K.K.M. contributed equally to this work.

Table 1. Mutant receptor constructs

Designation	Schematic representation
wt-STaR	
HA-wt-STaR	
FLAG-wt-STaR	
HA-kin$^-$	
FLAG-kin$^-$	
HA-cyc$^-$	
FLAG-cyc$^-$	
FLAG-ecd	
FLAG-D834A	

ECD TM KHD cyclase ▮ HA ▮ FLAG

ECD, extracellular domain; TM, transmembrane domain; KHD, kinase-homology domain; cyclase, guanylyl cyclase catalytic domain; HA, HA (12CA5) epitope; FLAG, FLAG epitope.

were transiently transfected with wt-STaR and/or mutant constructs using DEAE-dextran (15).

Guanylyl Cyclase Stimulation Assays. Synthetic ST (ST1b) was prepared using fluoren-9-ylmethoxycarbonyl chemistry as described (16). Cells transfected with either wt-STaR or mutant constructs were split into six-well plates. Forty-eight to 72 h later, cells were stimulated with ST, and extracts were then prepared as described (8). Samples were acetylated and analyzed for cGMP concentration by RIA (Biomedical Technologies, Stoughton, MA).

Coimmunoprecipitation and Western Blotting. Forty-eight hours after cotransfection, cells in 100-mm plates were washed twice with PBS and lysed in 1 ml of cold lysis buffer [20 mM Hepes, pH 7.5/250 mM NaCl/1% Triton X-100/1% sodium deoxycholate/aprotinin (20 μg/ml)]. After a 10-min incubation at 4°C, lysates were clarified by centrifugation, and supernates were incubated for 1 h at 4°C with 8 μg of anti-HA monoclonal antibody (mAb) (clone 12CA5; Boehringer Mannheim). Protein A-Sepharose CL-4B (Pharmacia) was then added for an additional hour (50 μl of 50% suspension) to precipitate the immune complexes. Protein A beads were washed four times with cold lysis buffer. The immunoprecipitated samples were heated at 75°C for 5 min in 40 μl of SDS sample buffer. Samples were analyzed by Western blot with anti-HA (8 μg/ml) or anti-FLAG M2 (20 μg/ml; IBI) mAb; followed by ECL detection (Amersham).

RESULTS

Functional Activity of Cytoplasmic Deletion Mutants. The kinase-homology domain of STaR is a 310-aa region that is interposed between the transmembrane and guanylyl cyclase domains. It has weak homology to the protein kinase consensus sequence but lacks both the invariant lysine and the nucleotide-binding motif that are essential for protein kinase activity (17, 18). The functional role of the kinase-homology domain of STaR is unknown. To determine whether it participates in the regulation of signaling, a deletion mutant (kin$^-$) was prepared, and the effects of this deletion on receptor signaling were tested. The deletion mutant was transiently expressed in COS cells, and the recombinant proteins were then analyzed for guanylyl cyclase activity in both the presence and absence of ST. wt-STaR was transfected in parallel as a positive control, and equivalent levels of the expressed recombinant wt-STaR (~135 kDa) and kin$^-$ (~100 kDa) proteins were confirmed by Western blot (data not shown). Cells transfected with wt-STaR produced low quantities of cGMP (0.4 ± 0.2 pmol/mg), which were comparable to those of vector-transfected cells (Fig. 1). After stimulation with saturating concentrations of ST (4 μM) (Fig. 1), wt-STaR transfectants showed an ~90-fold increase in intracellular concentrations of cGMP (34.8 ± 6.6 pmol/mg), consistent with previously published studies (3). When the kin$^-$ mutant was expressed in COS cells, levels of cGMP were high in the absence of ST (34.3 ± 6.0 pmol/mg) and could not be further increased by incubation with toxin (35.6 ± 6.2 pmol/mg). This indicates that guanylyl cyclase in the kin$^-$ mutants is constitutively activated, resulting in a ligand-independent production of cGMP that is equal in magnitude to that seen for the maximally stimulated wt-STaR. Thus, the kinase-homology domain has an autoinhibitory effect on guanylyl cyclase activity; its removal mimics the effects of ligand stimulation.

The effects of other large deletions of the cytoplasmic domains on ligand-induced signaling are shown in Fig. 1. Deletion of the C-terminal 368 aa (cyc$^-$) containing the consensus sequence for the guanylyl cyclase catalytic site predictably abolished ST-inducible cGMP production. Also, as expected, a construct containing only the extracellular and transmembrane domains (ecd) was inactive. Epitope-tagged derivatives of these two constructs were used in the studies below to delineate their potential contributions to receptor oligomerization and to toxin-mediated receptor activation.

Analysis of Receptor–Receptor Interactions Using Epitope-Tagged cDNA Constructs. Oligomerization is required for signal transduction by many receptors with single transmembrane-spanning domains. The best characterized examples include the tyrosine kinase and cytokine receptor families, which undergo ligand-induced dimerization through their extracellular domains. The importance of oligomeric structure for ST receptor signaling is unknown.

To facilitate our study of these potential oligomeric interactions, we inserted HA and FLAG epitope tags into the N-termini of wt-STaR and mutant receptor cDNAs. Both HA- and FLAG-wt-STaR constructs had similar affinities (EC_{50}) and efficacies (V_{max}) as the unmodified wt-STaR; intracellular cGMP levels increased ~90-fold in the presence of 1 μM ST, and the EC_{50} value for this response was ~150 nM ST. Epitope-tagged kin$^-$ constructs showed the same constitutive activity as the original kin$^-$ mutant. Having established that these N-terminal modifications do not affect the cellular expression or pharmacologic properties of STaR, we proceeded to use these constructs to study receptor–receptor interactions through cotransfection strategies.

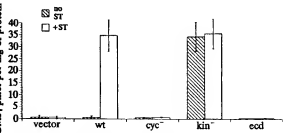

FIG. 1. cGMP production in response to ST by wt-STaR and related mutant receptors. COS cells transfected with the indicated cDNAs (8 μg) were treated with 4 μM ST (+ST) or PBS (no ST) for 10 min at 37°C. Intracellular cGMP levels were then determined as described in *Methods*. Results are expressed as the mean ± SEM of at least four separate determinations. wt, wt-STaR.

To determine whether the receptor forms oligomeric complexes, we cotransfected COS cells with equimolar quantities of FLAG- and HA-wt-STaR. Cells were lysed and immunoprecipitated using anti-HA mAb. Equal samples of the immunoprecipitates were analyzed by Western blot (Fig. 2*A*) probed with either anti-FLAG M2 or anti-HA mAb (positive control). The specificity of these immunoprecipitated proteins was verified using controls in which the anti-HA mAb was preincubated with HA peptide prior to immunoprecipitation (Fig. 2 *A–C*, peptide +). Western blot with anti-FLAG mAb revealed a specific band of ~135 kDa, indicating that the full-length receptors *do* physically interact. These data also demonstrate that the cellular receptor exists in an oligomeric state in the absence of ligand and that exposure to toxin does not alter the ratio of oligomeric forms.

The data of Fig. 2*A* suggest that ligand treatment does not change the proportion of aggregated receptors. To further address this question, we used epitope-tagged kin⁻ mutants, which are in a constitutively activated state. We hypothesized that coimmunoprecipitation studies would yield a negative result if a monomeric or dissociated state were required for cyclase activation. We cotransfected the HA- and FLAG-kin⁻ mutants and subjected them to coimmunoprecipitation to test whether these receptors were aggregated. These results shown in Fig. 2*B* indicate that like wt-STaR, the kin⁻ mutants physically interact in a ligand-independent fashion, demonstrating that the kinase-homology domain is not required for oligomerization.

Next, we asked whether a molecule lacking the cyclase domain (cyc⁻) was capable of oligomerization to wt-STaR. Ligand-independent coimmunoprecipitation of the cyc⁻ and wt-STaR is observed (Fig. 2*C*), indicating that these proteins can interact. cyc⁻ mutants are also able to form homooligomers (data not shown). In contrast, FLAG-ecd, a truncated receptor consisting only of the extracellular and transmembrane domains with a small cytoplasmic tail, failed to coimmunoprecipitate with wt-STaR (data not shown). Thus, the individual domains do not appear to interact with the full-length receptor. There is sufficient interaction, however, when the extracellular and kinase-homology domains are in cis to promote a physical interaction between these receptor mutants. It therefore seems likely that oligomerization of the STaR is the result of interactions in multiple domains (Fig. 2).

Demonstration of a Dominant-Negative Phenotype. The above studies established that oligomerization of STaR is a ligand-independent process and that there are several regions within the receptor molecule that facilitate this self-association. We next asked whether oligomerization is *necessary* for ligand-induced guanylyl cyclase activation and, if so, which region(s) of the receptor are critical for signal transduction. First, we cotransfected the receptor mutants, ecd and cyc⁻ (which both lack a guanylyl cyclase domain), with wt-STaR. Neither of these mutants inhibited ST-mediated cGMP production, even when transfected at a 30-fold molar excess over wt-STaR. Although there are potential regions for physical association in the cyc⁻ mutant (Fig. 2), these were not sufficient to produce a dominant-negative effect.

We next hypothesized that interaction within the cyclase domain could be important for receptor activation. Recently, Yuen *et al.* (19) have created dominant-negative mutants of the nitric oxide-sensitive soluble guanylyl cyclase. This was accom-

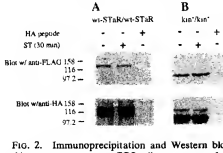

FIG. 2. Immunoprecipitation and Western blotting of wt-STaR and its mutant constructs. COS cells were cotransfected with wt-STaR or mutant cDNA (4 μg each). At 48–72 h posttransfection, they were incubated for 30 min at 37°C in the presence (+) or absence (–) of 1 μM ST. Cell lysates were immunoprecipitated with anti-HA mAb (peptide –) or with mAb preabsorbed to HA peptide (100 μg) for 1 h at 4°C (peptide +). Samples were subsequently incubated on immunoblots with anti-FLAG or anti-HA mAb. (*A*) Cells were transfected with HA- and FLAG-wt-STaR. (*B*) Cells were transfected with HA- and FLAG-kin⁻. (*C*) Cells were transfected with HA-wt-STaR and FLAG-cyc⁻.

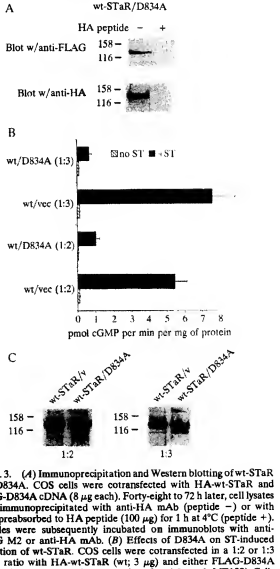

FIG. 3. (*A*) Immunoprecipitation and Western blotting of wt-STaR and D834A. COS cells were cotransfected with HA-wt-STaR and FLAG-D834A cDNA (8 μg each). Forty-eight to 72 h later, cell lysates were immunoprecipitated with anti-HA mAb (peptide –) or with mAb preabsorbed to HA peptide (100 μg) for 1 h at 4°C (peptide +). Samples were subsequently incubated on immunoblots with anti-FLAG M2 or anti-HA mAb. (*B*) Effects of D834A on ST-induced activation of wt-STaR. COS cells were cotransfected in a 1:2 or 1:3 molar ratio with HA-wt-STaR (wt; 3 μg) and either FLAG-D834A cDNA (D834A; 6 or 9 μg) or vector (vec; 3 or 4.5 μg of pME18S). Cells were stimulated with 185 nM ST for 9 min. Experiments were performed in triplicate, and cGMP values are expressed as pmol per mg of protein per min. Error bars indicate SEM. (*C*) Expression of wt-STaR in COS cells cotransfected with wt-STaR/D834A and wt-STaR/vector (wt-STaR/v). COS cells were transfected with appropriate cDNA as described in *B*. Cell lysates were immunoprecipitated with anti-HA mAb and subsequently analyzed on immunoblots with anti-HA mAb. Equal expression of wt-STaR in both cotransfections was confirmed by scanning densitometry (data not shown).

plished by disabling the catalytic function of one of the heterodimeric components through a conserved aspartic acid near the catalytic site. Based on this finding, we reasoned that if this conserved residue could similarly inactivate cyclase catalytic activity, we could then test for a dominant-negative phenotype. The D834A construct was expressed in COS cells and showed similar levels of cell surface expression as wt-STaR by immunofluorescence (data not shown). When D834A-transfected cells were incubated with 1 μM ST, the cGMP response was absent, indicating that this point mutation inactivated catalytic function. Nevertheless, when the FLAG-D834A construct was cotransfected with HA-wt-STaR, heterodimers were formed (Fig. 3*A*).

Having now established that there is interaction between wt-STaR and the catalytically inactive mutant D834A, we asked whether this interaction interferes with ST-induced cyclase activation, to produce a dominant-negative phenotype. Control cells cotransfected with wt-STaR and a 2-fold excess of empty vector were activated ~50-fold in the presence of 185 nM ST (~EC_{75}). Cotransfection with a 2-fold excess of D834A inhibited ST-mediated cyclase activation by a factor of 2.5. Cotransfection with a 3-fold excess of D834A more effectively inhibited ST-induced cyclase activation by a factor of ~8 (Fig. 3*B*). In experiments using a 1:10 ratio of wt-STaR/D834A, 5 μM ST induced only a 2.9-fold increase in cGMP, as compared to 30.3-fold for wt-STaR/vector-transfected controls, representing ~11-fold inhibition. Similar results were obtained in the presence of 185 nM and 1 μM ST, further supporting a dominant-negative effect (data not shown). Immunoprecipitation with anti-HA mAb confirmed equal expression of HA-wt-STaR when cotransfected with either vector or FLAG-D834A (Fig. 3*C*). Excess expression of the FLAG-D834A was confirmed with anti-FLAG mAb using the same methodology (data not shown). Thus, the dominant-negative phenotype of D834A suggests that at least two functional catalytic domains are required for ligand-induced activation. The results of the above cotransfection experiments are summarized in Table 2.

DISCUSSION

The guanylyl cyclase receptors are a diverse family of transmembrane receptors found in mammals as well as in lower eukaryotes. These receptors have distinct ligand specificities, and, accordingly, they regulate diverse physiological processes through ligand-specific production of intracellular cGMP. STaR is predominantly localized to the mammalian intestinal epithelium (16, 20); the diarrheal disease associated with enterotoxigenic *E. coli* infection results from toxin-mediated activation of the receptor.

In this report we begin to elucidate the molecular mechanisms leading to signal transduction by STaR. We show that deletion of the kinase-homology domain of STaR leads to constitutive, ligand-independent production of cGMP, equivalent to that of a maximally stimulated receptor. This indicates that the domain has an autoinhibitory effect on receptor function. Elegant studies by Chinkers and Garbers (21) previously demonstrated that the kinase-homology domain of the ANP-A receptor acts as a negative regulator of signaling. Further analysis by Koller *et al.* (17) demonstrated that the kinase-homology domain of STaR cannot effectively regulate ligand activation of the ANP receptor. Based on this finding and on the limited homology of their kinase-homology domains (~35%), it was proposed that there may be differences in the signaling mechanisms of ST and ANP receptors (17). Our results suggest that despite marked differences in their primary sequences, ST and ANP receptors may have similar three-dimensional structures resulting in a common regulatory motif.

The quarternary structure of STaR has not yet been fully elucidated. Here we have shown that it exists in cultured cells as an oligomer and that this oligomeric state is ligand independent. The natriuretic peptide receptor, GC-A, also has an oligomeric structure, and the relationship of its oligomeric state to hormone binding is still under investigation (22–24). Radiation inactivation data suggest that STaR is a heterotrimer made up of two full-length monomers plus an N-terminal proteolytic fragment (25). Our coimmunoprecipitation studies in COS cells reveal only a single band of ~135 kDa and suggest that the receptor is a homooligomer, as has been proposed for GC-A (22, 23). Using coimmunoprecipitation, we have demonstrated an interaction between cyc^- and wt-STaR. Despite this interaction, a dominant-negative phenotype was not ob-

Table 2. Summary of mutant constructions, receptor-receptor interaction, functional phenotypes

Constructs transfected	Functional phenotype*	Oligomerization†
wt-STaR	wt	+
kin–	Constitutive	+
cyc–	Inactive	+
wt-STaR ecd	wt	–
wt-STaR cyc–	wt	+
wt-STaR D834A	Dominant negative	+

The expression of *all* transfected constructs was confirmed by Western blot. All transfected constructs containing the extracellular domain displayed similar specific binding to ^{125}I-labeled ST. See Table 1 for the key.

*wt phenotype = ST-induced guanylyl cyclase activation.

†Oligomerization was determined by cotransfection with equimolar amounts of HA and FLAG epitope-tagged receptor followed by coimmunoprecipitation as described in *Methods*.

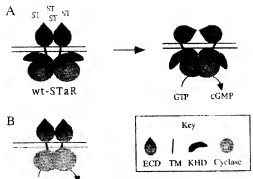

FIG. 4. Schematic representation of wt-STaR and kin⁻ mutant. This model illustrates a potential model for signal transduction by wt-STaR (*A*) and the kin⁻ mutant (*B*). ECD, extracellular domain; TM, transmembrane domain; KHD, kinase-homology domain; Cyclase, guanylyl cyclase catalytic domain.

served in transfections using as much as a 30-fold molar excess of cyc⁻. This could be due to relatively weak interactions between wt-STaR and cyc⁻ proteins, resulting in preferential oligomerization of the wt-STaR/wt-STaR homodimer over the wt-STaR/cyc⁻ heterodimer. Weaker interactions involving only the extracellular and kinase-homology domains may serve to stabilize the oligomer structure or facilitate translation or intracellular trafficking of the receptor (26, 27). *In vitro* translation studies are needed to further elucidate the stoichiometry and functional significance of these intermolecular interactions.

We prepared a point mutant at Asp-834 (D834A), a residue that is highly conserved in all guanylyl cyclases. This aspartic acid to alanine mutation abolishes toxin-mediated cyclase activation and produces a dominant-negative phenotype when coexpressed with wt-STaR. These data suggest that the cyclase domain is the critical region for functional interaction of receptor subunits. Yuen *et al.* (19) have recently demonstrated that a mutation of a homologous residue in the α subunit of nitric oxide-sensitive guanylyl cyclase also exerts a dominant-negative effect on activation of the α1/β1 heterodimer. Our findings suggest that despite the structural and functional differences between STaR and the soluble cyclase, both require at least two functional catalytic domains for activation.

One mechanism for the dominant-negative effect may be related to the requirement of a shared catalytic site (19) or to stabilization of the catalytic site by the interaction of these monomers. Shared sites composed of monomers that each contribute different residues to the catalytic process have been demonstrated for phosphofructokinase (28). An alternative hypothesis is that the binding of toxin stimulates oligomerization within the cyclase domain and that D834A causes a nonproductive interaction in this critical region. Asp-834 is within the guanylyl cyclase domain; however, additional sequence analysis suggests that this residue also lies within a putative helix–turn–helix motif. It is possible that this region represents an additional point of interaction that is necessary for productive signaling. Other mutations to this potential dimerization motif will help to elucidate this potential role.

Fig. 4*A* incorporates our findings into a potential model for signal transduction. The binding of toxin is hypothesized to induce a conformational change that is transduced across the membrane. This removes the inhibitory effects of the kinase homology domain and permits productive interaction of the cyclase domains. Fig. 4*B* depicts a mechanism for the constitutive activity that is observed when the kinase-homology domain is removed. In this model, the cyclase domains are no longer under the autoinhibitory control of these absent domains.

In summary, our data demonstrate a ligand-independent oligomeric state for STaR. The dominant-negative phenotype of the D834A mutant suggests that interaction of at least two cyclase domains is necessary for toxin-induced cyclase activation and that the interacting monomers must both be catalytically functional. We demonstrate that the kinase-homology domain has a negative regulatory effect on toxin-mediated signal transduction. This work elucidates a signal transduction paradigm for STaR, which may also be relevant to other guanylyl cyclase receptors.

Note. After this manuscript was submitted, Thompson and Garbers (29) described a dominant-negative mutant of the ANP receptor.

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EXHIBIT 8

US005518888A

United States Patent [19]

Waldman

[11] Patent Number: 5,518,888

[45] Date of Patent: May 21, 1996

[54] ST RECEPTOR BINDING COMPOUNDS AND METHODS OF USING THE SAME

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[21] Appl. No.: 141,892

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[58] Field of Search 424/1.49, 1.69, 424/9, 85.8, 9.1, 9.341; 435/7.21, 7.23; 530/388.22, 388.26, 388.8, 389.7, 324, 325, 326, 327

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(List continued on next page.)

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[57] ABSTRACT

Conjugated compounds which comprises an ST receptor binding moiety and a radiostable active moiety are disclosed. Pharmaceutical compositions comprising a pharmaceutically acceptable carrier or diluent, and a conjugated compound which comprises an ST receptor binding moiety and a radiostable active moiety or an ST receptor binding moiety and a radioactive active moiety are disclosed. Methods of treating an individual suspected of suffering from metastasized colorectal cancer comprising the steps of administering to said individual a pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent, and a therapeutically effective amount of a conjugated compound which comprises an ST receptor binding moiety and a radiostable active moiety or an ST receptor binding moiety and a radiostable active moiety are disclosed. Methods of radioimaging metastasized colorectal cancer cells comprising the steps of first administering to an individual suspected of having metastasized colorectal cancer cells, a pharmaceutical composition that comprises a pharmaceutically acceptable carrier or diluent, and conjugated compound that comprises an ST receptor binding moiety and a radioactive active moiety wherein the conjugated compound is present in an amount effective for diagnostic use in humans suffering from colorectal cancer and then detecting the localization and accumulation of radioactivity in the individual's body are disclosed.

22 Claims, No Drawings

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ST RECEPTOR BINDING COMPOUNDS AND METHODS OF USING THE SAME

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FIELD OF THE INVENTION

The present invention relates to compounds which comprise a receptor ligand moiety conjugated to an active agent. More particularly, the present invention relates to compounds which comprise a moiety that binds to the ST receptor conjugated to a therapeutic or imaging moiety.

BACKGROUND OF THE INVENTION

Colorectal cancer is the third most common neoplasm worldwide and the second most common in the United States, representing about 15% of the newly diagnosed cases of cancer in the United States. The large intestine or large bowel is the third leading site for the development of new cancer and is diagnosed in about 150,000 patients each year. Colorectal cancer is the second leading cause of cancer-related deaths and is responsible for about 12% of cancer deaths in the United States. The mortality rate of newly diagnosed large bowel cancer approaches 50% and there has been little improvement over the past 40 years. Most of this mortality reflects local, regional and distant metastases. About thirty percent of patients with colorectal cancer have unresectable disease at presentation and about 40% develop metastases during the course of their disease. Distant metastatic disease is seen in liver (about 12%), lung (about 3%), bone (about 0.9%), brain (about 0.7%), nodes (about 4%), and peritoneum (about 2%) at the time of initial diagnosis. In 1987, the large bowel cancers found regionally or at distant sites at the time of diagnosis were about 26% and about 18%, respectively.

Surgery is the mainstay of treatment for colorectal cancer but recurrence is frequent. Colorectal cancer has proven resistant to chemotherapy, although limited success has been achieved using a combination of 5-fluorouracil and levamisole. Surgery has had the largest impact on survival and, in some patients with limited disease, achieves a cure. However, surgery removes bulk tumor, leaving behind microscopic residual disease which ultimately results in recrudescence. Overall recurrence rates for colonic tumors are about 33% and for rectal cancer about 42%. Of these recurrences, about 9% are local, about 13% are systemic metastatic disease, and the remaining 88% are a combination of local and systemic disease. Fifty percent of patients with recurrent colorectal cancer have hepatic metastases.

Early detection of primary, metastatic, and recurrent disease can significantly impact the prognosis of individuals suffering from colorectal cancer. Large bowel cancer diagnosed at an early stage has a significantly better outcome than that diagnosed at more advanced stages. The 5 year relative survival rates for patients with regional or distant metastases are 48% and 5%, compared with 90% and 77% for disease which is in situ or local, respectively, at the time of diagnosis. Similarly, diagnosis of metastatic or recurrent disease earlier potentially carries with it a better prognosis.

Although current radiotherapeutic agents, chemotherapeutic agents and biological toxins are potent cytotoxins, they do not discriminate between normal and malignant cells, producing adverse effects and dose-limiting toxicities. Over the past decade, a novel approach has been employed to more specifically target agents to tumor cells, involving the conjugation of an active agent to molecules which binds preferentially to antigens that exist predominantly on tumor cells. These conjugates can be administered systemically and specifically bind to the targeted tumor cells. Theoretically, targeting permits uptake by cells of cytotoxic agents at concentrations which do not produce serious toxicities in normal tissues. Also, selective binding to targeted tumor cells facilitates detection of occult tumor and is therefore useful in designing imaging agents. Molecular targeting predominantly has employed monoclonal antibodies generated to antigens selectively expressed on tumor cells.

Immunoscintigraphy using monoclonal antibodies directed at tumor-specific markers has been employed to diagnose colorectal cancer. Monoclonal antibodies against carcinoembryonic antigen (CEA) labeled with ^{99m}Technetium identified 94% of patients with recurrent tumors. Similarly, ^{111}Indium-labeled anti-CEA monoclonal antibodies successfully diagnosed 85% of patients with recurrent colorectal carcinoma who were not diagnosed by conventional techniques. ^{125}Iodine-labeled antibodies have been effective in localizing more than 80% of the pathologically-confirmed recurrences by intraoperative gamma probe scanning.

Monoclonal antibodies have also been employed to target specific therapeutic agents in colorectal cancer. Preclinical studies demonstrated that anti-CEA antibodies labeled with ^{90}Yitrium inhibited human colon carcinoma xenografts in nude mice. Antibodies generated to colorectal cancer cells and coupled to mitomycin C or neocarzinostatin demonstrated an anti-tumor effect on human colon cancer xenografts in nude mice and 3 patients with colon cancer. Similar results in animals were obtained with monoclonal antibodies conjugated to ricin toxin A chain.

Due to the sensitivity, specificity, and adverse-effect profile of monoclonal antibodies, the results obtained using monoclonal antibody-based therapeutics have shown them to be less than ideal targeting tools. Although monoclonal antibodies have been generated to antigens selectively expressed on tumors, no truly cancer-specific antibody has been identified. Most antigens expressed on neoplastic cells appear to be quantitatively increased in these compared to normal cells but the antigens are nonetheless often present in normal cells. Thus, antibodies to such determinants can react with non-neoplastic tissues, resulting in significant toxicities. Also, antibodies are relatively large molecules and consequently, often evoke an immune response in patients. These immune responses can result in significant toxicities in patients upon re-exposure to the targeting agents and can prevent targeting by the monoclonal due to immune complex formation with degradation and excretion. Finally, binding of antibodies to tumor cells may be low and targeted agents may be delivered to cells in quantities insufficient to achieve detection or cytotoxicity.

There remains a need for compositions which can specifically target metastasized colorectal cancer cells. There is a need for imaging agents which can specifically bind to metastasized colorectal cancer cells. There is a need for improved methods of imaging metastasized colorectal cancer cells. There is a need for therapeutic agents which can specifically bind to metastasized colorectal cancer cells. There is a need for improved methods of treating individuals who are suspected of suffering from colorectal cancer cells,

especially individuals who are suspected of suffering from metastasis of colorectal cancer cells.

SUMMARY OF THE INVENTION

The present invention relates to conjugated compounds which comprises an ST receptor binding moiety and a radiostable active moiety.

The present invention relates to a pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent, and a conjugated compound which comprises an ST receptor binding moiety and a radiostable active moiety.

The present invention relates to a method of treating an individual suspected of suffering from metastasized colorectal cancer comprising the steps of administering to said individual a pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent, and a therapeutically effective amount of a conjugated compound which comprises an ST receptor binding moiety and a radiostable active moiety.

The present invention relates to a pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent, and conjugated compound that comprises an ST receptor binding moiety and a radioactive active moiety wherein the conjugated compound is present in an amount effective for therapeutic or diagnostic use in humans suffering from colorectal cancer.

The present invention relates to a method of radioimaging metastasized colorectal cancer cells comprising the steps of first administering to an individual suspected of having metastasized colorectal cancer cells, a pharmaceutical composition that comprises a pharmaceutically acceptable carrier or diluent, and conjugated compound that comprises an ST receptor binding moiety and a radioactive active moiety wherein the conjugated compound is present in an amount effective for diagnostic use in humans suffering from colorectal cancer and then detecting the localization and accumulation of radioactivity in the individual's body.

The present invention relates to a method of treating an individual suspected of suffering from metastasized colorectal cancer comprising the steps of administering to said individual a pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent, and a therapeutically effective amount of a conjugated compound which comprises an ST receptor binding moiety and a radioactive active moiety.

DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

As used herein, the terms "ST" and "native ST" are used interchangeably and are meant to refer to heat-stable toxin (ST) which is a peptide produced by *E. coli*, as well as other organisms. STs are naturally occurring peptides which 1) are naturally produced by organisms, 2) which bind to the ST receptor and 3) which activate the signal cascade that mediates ST-induced diarrhea.

As used herein, the term "ST receptor" is meant to refer to the receptors found on colorectal cells, including local and metastasized colorectal cancer cells, which bind to ST. In normal individuals, ST receptors are found exclusively in cells of intestine, in particular in cells in the duodenum, small intestine (jejunum and ileum), the large intestine, colon (cecum, ascending colon, transverse colon, descending colon and sigmoid colon) and rectum.

As used herein, the term "ST receptor ligand" is meant to refer to compounds which specifically bind to the ST receptor. ST is an ST receptor ligand. An ST receptor ligand may be a peptide or a non-peptide.

As used herein, the term "ST receptor binding peptide" is meant to refer to ST receptor ligands that are peptides.

As used herein, the term "ST peptides" is meant to refer to ST receptor binding peptides selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5–54 and fragments and derivatives thereof.

As used herein, the term "fragment" is meant to refer to peptide a) which has an amino acid sequence identical to a portion of an ST receptor binding peptide and b) which is capable of binding to the ST receptor.

As used herein, the term "derivative" is meant to refer to a peptide a) which has an amino acid sequence substantially identical to at least a portion of an ST receptor binding peptide and b) which is capable of binding to the ST receptor.

As used herein, the term "substantially identical" is meant to refer to an amino acid sequence that is the same as the amino acid sequence of an ST peptide except some of the residues are deleted or substituted with conservative amino acids or additional amino acids are inserted.

As used herein, the term "active agent" is meant to refer to compounds that are therapeutic agents or imaging agents.

As used herein, the term "radiostable" is meant to refer to compounds which do not undergo radioactive decay; i.e. compounds which are not radioactive.

As used herein, the term "therapeutic agent" is meant to refer to chemotherapeutics, toxins, radiotherapeutics, targeting agents or radiosensitizing agents.

As used herein, the term "chemothrapeutic" is meant to refer to compounds that, when contacted with and/or incorporated into a cell, produce an effect on the cell including causing the death of the cell, inhibiting cell division or inducing differentiation.

As used herein, the term "toxin" is meant to refer to compounds that, when contacted with and/or incorporated into a cell, produce the death of the cell.

As used herein, the term "radiotherapeutic" is meant to refer to radionuclides which when contacted with and/or incorporated into a cell, produce the death of the cell.

As used herein, the term "targeting agent" is meant to refer compounds which can be bound by and or react with other compounds. Targeting agents may be used to deliver chemotherapeutics, toxins, enzymes, radiotherapeutics, antibodies or imaging agents to cells that have targeting agents associated with them and/or to convert or otherwise transform or enhance coadministered active agents. A targeting agent may include a moiety that constitutes a first agent that is localized to the cell which when contacted with a second agent either is converted to a third agent which has a desired activity or causes the conversion of the second agent into an agent with a desired activity. The result is the localized agent facilitates exposure of an agent with a desired activity to the metastasized cell.

As used herein, the term "radiosensitizing agent" is meant to refer to agents which increase the susceptibility of cells to the damaging effects of ionizing radiation. A radiosensitizing agent permits lower doses of radiation to be administered and still provide a therapeutically effective dose.

As used herein, the term "imaging agent" is meant to refer to compounds which can be detected.

As used herein, the term "ST receptor binding moiety" is meant to refer to the portion of a conjugated compound that constitutes an ST receptor ligand.

As used herein, the term "active moiety" is meant to refer to the portion of a conjugated compound that constitutes an active agent.

As used herein, the terms "conjugated compound" and "conjugated composition" are used interchangeably and meant to refer to a compound which comprises an ST receptor binding moiety and an active moiety and which is capable of binding to the ST receptor. Conjugated compounds according to the present invention comprise a portion which constitutes an ST receptor ligand and a portion which constitutes an active agent. Thus, conjugated compounds according to the present invention are capable of specifically binding to the ST receptor and include a portion which is a therapeutic agent or imaging agent. Conjugated compositions may comprise crosslinkers and/or molecules that serve as spacers between the moieties.

As used herein, the terms "crosslinker", "crosslinking agent", "conjugating agent", "coupling agent", "condensation reagent" and "bifunctional crosslinker" are used interchangeably and are meant to refer to molecular groups which are used to attach the ST receptor ligand and the active agent to thus form the conjugated compound.

As used herein, the term "colorectal cancer" is meant to include the well-accepted medical definition that defines colorectal cancer as a medical condition characterized by cancer of cells of the intestinal tract below the small intestine (i.e. the large intestine (colon), including the cecum, ascending colon, transverse colon, descending colon, and sigmoid colon, and rectum). Additionally, as used herein, the term "colorectal cancer" is meant to further include medical conditions which are characterized by cancer of cells of the duodenum and small intestine (jejunum and ileum). The definition of colorectal cancer used herein is more expansive than the common medical definition but is provided as such since the cells of the duodenum and small intestine also contain ST receptors and are therefore amenable to the methods of the present invention using the compounds of the present invention.

As used herein, the term "metastasis" is meant to refer to the process in which cancer cells originating in one organ or part of the body relocate to another part of the body and continue to replicate. Metastasized cells subsequently form tumors which may further metastasize. Metastasis thus refers to the spread of cancer from the part of the body where it originally occurs to other parts of the body. The present invention relates to methods of delivering active agents to metastasized colorectal cancer cells.

As used herein, the term "metastasized colorectal cancer cells" is meant to refer to colorectal cancer cells which have metastasized; colorectal cancer cells localized in a part of the body other than the duodenum, small intestine (jejunum and ileum), large intestine (colon), including the cecum, ascending colon, transverse colon, descending colon, and sigmoid colon, and rectum.

ST, which is produced by *E. coli*, as well as other organisms, is responsible for endemic diarrhea in developing countries and travelers diarrhea. ST induces intestinal secretion by binding to specific receptors, ST receptors, in the apical brush border membranes of the mucosal cells lining the intestinal tract. Binding of ST to ST receptors is non-covalent and occurs in a concentration-dependent and saturable fashion. Once bound, ST-ST receptor complexes appear to be internalized by intestinal cells, i.e. transported from the surface into the interior of the cell. Binding of ST to ST receptors triggers a cascade of biochemical reactions in the apical membrane of these cells resulting in the production of a signal which induces intestinal cells to secrete fluids and electrolytes, resulting in diarrhea.

ST receptors are unique in that they are only localized in the apical brush border membranes of the cells lining the intestinal tract. Indeed, they are not found in any other cell type in placental mammals. In addition, ST receptors are almost exclusively localized to the apical membranes, with little being found in the basolateral membranes on the sides of intestinal cells.

Mucosal cells lining the intestine are joined together by tight junctions which form a barrier against the passage of intestinal contents into the blood stream and components of the blood stream into the intestinal lumen. Therefore, the apical location of ST receptors isolates these receptors from the circulatory system so that they may be considered to exist separate from the rest of the body; essentially the "outside" of the body. Therefore, the rest of the body is considered "outside" the intestinal tract. Compositions administered "outside" the intestinal tract are maintained apart and segregated from the only cells which normally express ST receptors.

In individuals suffering from colorectal cancer, the cancer cells are often derived from cells that produce and display the ST receptor and these cancer cells continue to produce and display the ST receptor on their cell surfaces. Indeed, T84 cells, which are human colonic adenocarcinoma cells isolated from lung metastases, express ST receptors on their cell surface. Similarly, HT29glu-cells, which are human colonic adenocarcinoma cells, express receptors for ST. Thus, in individuals suffering from colorectal cancer, some metastasized intestinal cancer cells express ST receptors.

An effort was undertaken to determine the proportion of colorectal tumors which have the ST receptor. Sixteen colorectal cancer tumors, including ten local colorectal tumors and six metastasized tumors (3 liver, 1 lung, 1 lymphnode, 1 peritoneum), were tested and each possessed ST receptors. In each case, the affinity and density of receptors was amenable for targeting. That is, the cells possessed at least 10^4–10^6 receptors per cell and demonstrated an affinity of 10^{-7} or better (that is preferably between 10^{-8} to 10^{-9} or less; the lower number indicating a tighter bond, thus a higher affinity). Normal liver, lymphnode, peritoneum and lung cells were found not to possess ST receptors.

When such cancer cells metastasize, the metastasized cancer cells continue to produce and display the ST receptor. The expression of ST receptors on the surfaces of metastatic tumors provides a target for selective binding of conjugated compositions. ST receptors permit the absolutely specific targeting of therapeutic and diagnostic agents that are conjugated to ST receptor ligands to metastatic colorectal cancer cells.

The conjugated compositions of the present invention are useful for targeting cells that line the inner intestine wall including those cancer cells derived from such cells, particularly metastasized cancer cells derived from such cells. The conjugated compositions of the present invention which are administered outside the intestinal tract such as those administered in the circulatory system will remain segregated from the cells that line the intestinal tract and will bind only to cells outside the intestinal tract which are derived from the intestinal tract such as metastasized colorectal cells. The conjugated compositions will not bind to noncolorectal derived cells. Thus, the active moieties of conjugated compositions administered outside the intestinal tract are delivered to cells which are derived from the intestinal tract such

as metastasized colorectal cells but will not be delivered to any other cells.

Therapeutic and diagnostic pharmaceutical compositions of the present invention include conjugated compounds specifically targeted to metastatic disease. These conjugated compounds include ST receptor binding moieties which do not bind to cells of normal tissue in the body except cells of the intestinal tract since the cells of other tissues do not possess ST receptors. Unlike normal colorectal cells and localized colorectal cancer cells, metastasized colorectal cancer cells are accessible to substances administered outside the intestinal tract, for example administered in the circulatory system. The only ST receptors in normal tissue exist in the apical membranes of intestinal mucosa cells and these receptors are effectively isolated from the targeted cancer chemotherapeutics and imaging agents administered outside the intestinal tract by the intestinal mucosa barrier. Thus, metastasized colorectal cells may be targeted by conjugated compounds of the present invention by introducing such compounds outside the intestinal tract such as for example by administering pharmaceutical compositions that comprise conjugated compounds into the circulatory system.

One having ordinary skill in the art can readily identify individuals suspected of suffering from colorectal cancer and metastasized colorectal cells. In those individuals diagnosed with colorectal cancer, it is standard therapy to suspect metastasis and aggressively attempt to eradicate metastasized cells. The present invention provides pharmaceutical compositions and methods for imaging and thereby will more definitively diagnose metastasis. Further, the present invention provides pharmaceutical compositions comprising therapeutic agents and methods for specifically targeting and eliminating metastasized colorectal cancer cells. Further, the present invention provides pharmaceutical compositions that comprise therapeutics and methods for specifically eliminating colorectal cancer cells.

The pharmaceutical compositions which comprise conjugated compositions of the present invention may be used to diagnose or treat individuals suffering from localized colorectal tumors, that is primary or non-metastatic colorectal tumors if these have penetrated the basement membrane underlying the mucosa into the submucosa where there is abundant blood supply to which they have access. Penetration into the submucosa circumvents the mucosal barrier resulting in the ability of conjugated compositions introduced into the circulatory system to interact with these tumors.

The present invention relies upon the use of an ST receptor binding moiety in a conjugated composition. The ST receptor binding moiety is essentially a portion of the conjugated composition which acts as a ligand to the ST receptor and thus specifically binds to these receptors. The conjugated composition also includes an active moiety which is associated with the ST receptor binding moiety; the active moiety being an active agent which is either useful to image, target, neutralize or kill the cell.

According to the present invention, the ST receptor binding moiety is the ST receptor ligand portion of a conjugated composition. In some embodiments, the ST receptor ligand may be native ST.

Native ST has been isolated from a variety of organisms including *E. coli*, Yersinia, Enterobacter, and others. In nature, the toxins are generally encoded on a plasmid which can "jump" between different species. Several different toxins have been reported to occur in different species. These toxins all possess significant sequence homology, they all bind to ST receptors and they all activate guanylate cyclase, producing diarrhea.

ST has been both cloned and synthesized by chemical techniques. The cloned or synthetic molecules exhibit binding characteristics which are similar to native ST. Native ST isolated from *E. coli* is 18 or 19 amino acids in length. The smallest "fragment" of ST which retains activity is the 13 amino acid core peptide extending toward the carboxy terminal from cysteine 6 to cysteine 18 (of the 19 amino acid form). Analogues of ST have been generated by cloning and by chemical techniques. Small peptide fragments of the native ST structure which include the structural determinant that confers binding activity may be constructed. Once a structure is identified which binds to ST receptors, non-peptide analogues mimicking that structure in space are designed.

SEQ ID NO:1 discloses a nucleotide sequence which encodes 19 amino acid ST, designated ST Ia, reported by So and McCarthy (1980) *Proc. Natl. Acad. Sci. USA* 77:4011, which is incorporated herein by reference.

The amino acid sequence of ST Ia is disclosed in SEQ ID NO:2.

SEQ ID NO:3 discloses the amino acid sequence of an 18 amino acid peptide which exhibits ST activity, designated ST I*, reported by Chan and Giannella (1981) *J. Biol. Chem.* 256:7744, which is incorporated herein by reference.

SEQ ID NO:4 discloses a nucleotide sequence which encodes 19 amino acid ST, designated ST Ib, reported by Mosely et al. (1983) *Infect. Immun.* 39:1167, which is incorporated herein by reference.

The amino acid sequence of ST Ib is disclosed in SEQ ID NO:5.

A 15 amino acid peptide called guanylin which has about 50% sequence homology to ST has been identified in mammalian intestine (Currie, M. G. et al. (1992) *Proc. Natl. Acad Sci. USA* 89:947–951, which is incorporated herein by reference). Guanylin binds to ST receptors and activates guanylate cyclase at a level of about 10- to 100-fold less than native ST. Guanylin may not exist as a 15 amino acid peptide in the intestine but rather as part of a larger protein in that organ. The amino acid sequence of guanylin from rodent is disclosed as SEQ ID NO:6.

SEQ ID NO:7 is an 18 amino acid fragment of SEQ ID NO:2. SEQ ID NO:8 is a 17 amino acid fragment of SEQ ID NO:2. SEQ ID NO:9 is a 16 amino acid fragment of SEQ ID NO:2. SEQ ID NO:10 is a 15 amino acid fragment of SEQ ID NO:2. SEQ ID NO:11 is a 14 amino acid fragment of SEQ ID NO:2. SEQ ID NO:12 is a 13 amino acid fragment of SEQ ID NO:2. SEQ ID NO:13 is an 18 amino acid fragment of SEQ ID NO:2. SEQ ID NO:14 is a 17 amino acid fragment of SEQ ID NO:2. SEQ ID NO:15 is a 16 amino acid fragment of SEQ ID NO:2. SEQ ID NO:16 is a 15 amino acid fragment of SEQ ID NO:2. SEQ ID NO:17 is a 14 amino acid fragment of SEQ ID NO:2.

SEQ ID NO:18 is a 17 amino acid fragment of SEQ ID NO:3. SEQ ID NO:19 is a 16 amino acid fragment of SEQ ID NO:3. SEQ ID NO:20 is a 15 amino acid fragment of SEQ ID NO:3. SEQ ID NO:21 is a 14 amino acid fragment of SEQ ID NO:3. SEQ ID NO:22 is a 13 amino acid fragment of SEQ ID NO:3. SEQ ID NO:23 is a 17 amino acid fragment of SEQ ID NO:3. SEQ ID NO:24 is a 16 amino acid fragment of SEQ ID NO:3. SEQ ID NO:25 is a 15 amino acid fragment of SEQ ID NO:3. SEQ ID NO:26 is a 14 amino acid fragment of SEQ ID NO:3.

SEQ ID NO:27 is an 18 amino acid fragment of SEQ ID NO:5. SEQ ID NO:28 is a 17 amino acid fragment of SEQ

ID NO:5. SEQ ID NO:29 is a 16 amino acid fragment of SEQ ID NO:5, SEQ ID NO:30 is a 15 amino acid fragment of SEQ ID NO:5. SEQ ID NO:31 is a 14 amino acid fragment of SEQ ID NO:5. SEQ ID NO:32 is a 13 amino acid fragment of SEQ ID NO: 5. SEQ ID NO: 33 is an 18 amino acid fragment of SEQ ID NO:5. SEQ ID NO:34 is a 17 amino acid fragment of SEQ ID NO:5. SEQ ID NO:35 is a 16 amino acid fragment of SEQ ID NO:5. SEQ ID NO:36 is a 15 amino acid fragment of SEQ ID NO:5. SEQ ID NO:37 is a 14 amino acid fragment of SEQ ID NO:5.

SEQ ID NO:27, SEQ ID NO:31, SEQ ID NO:36 AND SEQ ID NO:37 are disclosed in Yoshimura, S., et al. (1985) *FEBS Lett.* 181:138, which is incorporated herein by reference.

SEQ ID NO:38, SEQ ID NO:39 and SEQ ID NO:40, which are derivatives of SEQ ID NO:3, are disclosed in Waldman, S. A. and O'Hanley, P. (1989) *Infect. Immun.* 57:2420, which is incorporated herein by reference.

SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44 and SEQ ID NO:45, which are a dcrivatvcs of SEQ ID NO:3, are disclosed in Yoshimura, S., et al. (1985) *FEBS Lett.* 181:138, which is incorporated herein by reference.

SEQ ID NO:46 is a 25 amino acid peptide derived from *Y. enterocolitica* which binds to the ST receptor.

SEQ ID NO:47 is a 16 amino acid peptide derived from *V. cholerae* which binds to the ST receptor. SEQ ID NO:47 is reported in Shimonishi, Y., et al. *FEBS Lett.* 215:165, which is incorporated herein by reference.

SEQ ID NO:48 is an 18 amino acid peptide derived from *Y. enterocolitica* which binds to the ST receptor. SEQ ID NO:48 is reported in Okamoto, K., et al. *Infec. Immun.* 55:2121, which is incorporated herein by reference.

SEQ ID NO:49, is a derivative of SEQ ID NO:5.

SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52 and SEQ ID NO:53 are derivatives.

SEQ ID NO:54 is the amino acid sequence of guanylin from human.

In some preferred embodiments, conjugated compounds comprise ST receptor binding moieties that comprise amino acid sequences selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5–54 and fragments and derivatives thereof.

Those having ordinary skill in the art can readily design and produce derivatives having substantially identical amino acid sequences of ST peptides with deletions and/or insertions and/or conservative substitutions of amino acids. For example, following what are referred to as Dayhof's rules for amino acid substitution (Dayhof, M. D. (1978) *Nat. Biomed. Res. Found.*, Washington, D.C. Vol. 5, supp. 3), amino acid residues in a peptide sequence may be substituted with comparable amino acid residues. Such substitutions are well-known and are based the upon charge and structural characteristics of each amino acid. Derivatives include fragments of ST receptor binding peptides with deletions and/or insertions and/or conservative substitutions.

In some embodiments, ST receptor binding peptides comprise D amino acids. As used herein, the term "D amino acid peptides" is meant to refer to ST receptor binding peptides, fragments or derivatives which comprise at least one and preferably a plurality of D amino acids which are capable of binding to the ST receptor. The use of D amino acid peptides is desirable as they are less vulnerable to degradation and therefore have a longer half-life. D amino acid peptides comprising mostly all or consisting of D amino acids may comprise amino acid sequences in the reverse order of ST receptor binding peptides which are made up of L amino acids.

In some embodiments, ST receptor binding peptides, including D amino acid peptides, are conformationally restricted to present and maintain the proper structural conformation for binding to the ST receptor. The compositions may comprise additional amino acid residues required to achieve proper three dimensional conformation including residues which facilitate circularization or desired folding.

It is preferred that the ST receptor ligand used as the ST receptor binding moiety be as small as possible. Thus it is preferred that the ST receptor ligand be a non-peptide small molecule or small peptide, preferably less than 25 amino acids, more preferably less than 20 amino acids. In some embodiments, the ST receptor ligand which constitute the ST receptor binding moiety of a conjugated composition is less than 15 amino acids. ST receptor binding peptide comprising less than 10 amino acids and ST receptor binding peptide less than 5 amino acids may be used as ST binding moieties according to the present invention. It is within the scope of the present invention to include larger molecules which serve as ST receptor binding moieties including, but not limited to molecules such as antibodies, FAbs and F(Ab)2s which specifically bind to ST receptor.

An assay may be used to test both peptide and nonpeptide compositions to determine whether or not they are ST receptor ligands or, to test conjugated compositions to determine if they possess ST receptor binding activity. Such compositions that specifically bind to ST receptors can be identified by a competitive binding assay. The competitive binding assay is a standard technique in pharmacology which can be readily performed by those having ordinary skill in the art using readily available starting materials. Competitive binding assays have been shown to be effective for identifying compositions that specifically bind to ST receptors. Briefly, the assay consists of incubating a preparation of ST receptors (e.g. intestinal membranes from rat intestine, human intestine, T84 cells) with a constant concentration (1×10^{-10}M to 5×10^{-10}M) of ^{125}I-ST (any ST receptor ligand such as native STs SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:5 may be used) and a known concentration of a test compound. As a control, a duplicate preparation of ST receptors are incubated with a duplicate concentration of ^{125}I-ST in the absence of test compound. Assays are incubated to equilibrium (2 hours) and the amount of ^{125}I-ST bound to receptors is quantified by standard techniques. The ability of the test compound to bind to receptors is measured as its ability to prevent (compete with) the ^{125}I-ST from binding. Thus, in assays containing the test compound which bind to the receptor, there will be less radioactivity associated with the receptors. This assay, which is appropriate for determining the abiliy of any molecule to bind to ST receptors, is a standard competitive binding assay which can be readily employed by those having ordinary skill in the art using readily available starting materials.

ST may be isolated from natural sources using standard techniques. Additionally, ST receptor binding peptides and conjugated compositions or portions thereof which are peptides may be prepared routinely by any of the following known techniques.

ST receptor binding peptides and conjugated compositions or portions thereof which are peptides may be prepared using the solid-phase synthetic technique initially described by Merrifield, in *J. Am. Chem. Soc.*, 15:2149–2154 (1963). Other peptide synthesis techniques may be found, for

example, in M. Bodanszky et al., (1976) *Peptide Synthesis*, John Wilcy & Sons, 2d Ed.; Kent and Clark-Lewis in *Synthetic Peptides in Biology and Medicine*, p. 295–358, eds. Alitalo, K., et al. Science Publishers, (Amsterdam, 1985); as well as other reference works known to those skilled in the art. A summary of peptide synthesis techniques may be found in J. Stuart and J. D. Young, *Solid Phase Peptide Synthelia*, Pierce Chemical Company, Rockford, Ill. (1984), which is incorporated herein by reference. The synthesis of peptides by solution methods may also be used, as described in *The Proteins*, Vol. II, 3d Ed., p. 105–237, Neurath, H. et al., Eds., Academic Press, New York, N.Y. (1976). Appropriate protective groups for use in such syntheses will be found in the above texts, as well as in J. F. W. McOmic, *Protective Groups in Organic Chemistry*, Plenum Press, New York, N.Y. (1973), which is incorporated herein by reference. In general, these synthetic methods involve the sequential addition of one or more amino acid residues or suitable protected amino acid residues to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid residue is protected by a suitable, selectively removable protecting group. A different, selectively removable protecting group is utilized for amino acids containing a reactive side group, such as lysine.

Using a solid phase synthesis as an example, the protected or derivatized amino acid is attached to an inert solid support through its unprotected carboxyl or amino group. The protecting group of the amino or carboxyl group is then selectively removed and the next amino acid in the sequence having the complementary (amino or carboxyl) group suitably protected is admixed and reacted with the residue already attached to the solid support. The protecting group of the amino or carboxyl group is then removed from this newly added amino acid residue, and the next amino acid (suitably protected) is then added, and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining terminal and side group protecting groups (and solid support) are removed sequentially or concurrently, to provide the final peptide. The peptide of the invention are preferably devoid of benzylated or methylbenzylated amino acids. Such protecting group moieties may be used in the course of synthesis, but they are removed before the peptides are used. Additional reactions may be necessary, as described elsewhere, to form intramolecular linkages to restrain conformation.

ST receptor binding peptides and conjugated compositions or portions thereof which are peptides may also be prepared by recombinant DNA techniques. Provision of a suitable DNA sequence encoding the desired peptide permits the production of the peptide using recombinant techniques now known in the art. The coding sequence can be obtained from natural sources or synthesized or otherwise constructed using widely available starting materials by routine methods. When the coding DNA is prepared synthetically, advantage can be taken of known codon preferences of the intended host where the DNA is to be expressed.

To produce an ST receptor binding peptide which occurs in nature, one having ordinary skill in the art can, using well-known techniques, obtain a DNA molecule encoding the ST receptor binding peptides from the genome of the organism that produces the ST receptor binding peptide and insert that DNA molecule into a commercially available expression vector for use in well-known expression systems.

Likewise, one having ordinary skill in the art can, using well known techniques, combine a DNA molecule that encodes an ST receptor binding peptide, such as SEQ ID NO:1 and SEQ ID NO:4, which can be obtained from the genome of the organism that produces the ST, with DNA that encodes a toxin, another active agent that is a peptide or additionally, any other amino acid sequences desired to be adjacent to the ST receptor binding peptide amino acid sequence in a single peptide and insert that DNA molecule into a commercially available expression vector for use in well-known expression systems.

For example, the commercially available plasmid pSE420 (Invitrogen, San Diego, Calif.) may be used for recombinant production in *E. coli*. The commercially available plasmid pYES2 (Invitrogen, San Diego, Calif.) may be used for production in *S. cerevisiae* strains of yeast. The commercially available MaxBac™ (Invitrogen, San Diego, Calif.) complete baculovirus expression system may be used for production in insect cells. The commercially available plasmid pcDNA I (Invitrogen, San Diego, Calif.) may be used for production in mammalian cells such as Chinese Hamster Ovary cells.

One having ordinary skill in the art may use these or other commercially available expression vectors and systems or produce vectors using well-known methods and readily available starting materials. Expression systems containing the requisite control sequences, such as promoters and polyadenylation signals, and preferably enhancers, are readily available and known in the art for a variety of hosts. See e.g., Sambrook et al., *Molecular Cloning a Laboratory Manual*, Second Ed. Cold Spring Harbor Press (1989). Thus, the desired proteins can be prepared in both prokaryotic and eukaryotic systems, resulting in a spectrum of processed forms of the protein.

The most commonly used prokaryotic system remains *E. coli*, although other systems such as *B. subtillis* and Pseudomonas are also useful. Suitable control sequences for prokaryotic systems include both constitutive and inducible promoters including the lac promoter, the trp promoter, hybrid promoters such as tac promoter, the lambda phage P1 promoter. In general, foreign proteins may be produced in these hosts either as fusion or mature proteins. When the desired sequences are produced as mature proteins, the sequence produced may be preceded by a methionine which is not necessarily efficiently removed. Accordingly, the peptides and proteins claimed herein may be preceded by an N-terminal Met when produced in bacteria. Moreover, constructs may be made wherein the coding sequence for the peptide is preceded by an operable signal peptide which results in the secretion of the protein. When produced in prokaryotic hosts in this matter, the signal sequence is removed upon secretion.

A wide variety of eukaryotic hosts are also now available for production of recombinant foreign proteins. As in bacteria, eukaryotic hosts may be transformed with expression systems which produce the desired protein directly, but more commonly signal sequences are provided to effect the secretion of the protein. Eukaryotic systems have the additional advantage that they are able to process introns which may occur in the genomic sequences encoding proteins of higher organisms. Eukaryotic systems also provide a variety of processing mechanisms which result in, for example, glycosylation, carboxy-terminal amidation, oxidation or derivatization of certain amino acid residues, conformational control, and so forth.

Commonly used eukaryotic systems include, but are not limited to, yeast, fungal cells, insect cells, mammalian cells, avian cells, and cells of higher plants. Suitable promoters are available which are compatible and operable for use in each of these host types as well as are termination sequences and

enhancers, as e.g. the baculovirus polyhedron promoter. As above, promoters can be either constitutive or inducible. For example, in mammalian systems, the mouse metallothionene promoter can be induced by the addition of heavy metal ions.

The particulars for the construction of expression systems suitable for desired hosts are known to those in the art. For recombinant production of the protein, the DNA encoding it is suitably ligated into the expression vector of choice and then used to transform the compatible host which is then cultured and maintained under conditions wherein expression of the foreign gene takes place. The protein of the present invention thus produced is recovered from the culture, either by lysing the cells or from the culture medium as appropriate and known to those in the art.

One having ordinary skill in the art can, using well-known techniques, isolate the protein that is produced.

According to the present invention, the active moiety may be a therapeutic agent or an imaging agent. One having ordinary skill in the art can readily recognize the advantages of being able to specifically target metastasized colorectal cells with an ST receptor ligand and conjugate such a ligand with many different active agents.

Chemotherapeutics useful as active moieties which when conjugated to an ST receptor binding moiety are specifically delivered to metastasized colorectal cells are typically, small chemical entities produced by chemical synthesis. Chemotherapeutics include cytotoxic and cytostatic drugs. Chemotherapeutics may include those which have other effects on cells such as reversal of the transformed state to a differentiated state or those which inhibit cell replication. Examples of chemotherapeutics include common cytotoxic or cytostatic drugs such as for example: methotrexate(amethopterin), doxorubicin(adrimycin), daunorubicin, cytosinarabinoside, etoposide, 5-4 fluorouracil, melphalan, chlorambucil, and other nitrogen mustards (e.g. cyclophosphamide), cis-platinum, vindesine (and other vinca alkaloids), mitomycin and bleomycin. Other chemotherapeutics include: purothionin (barley flour oligopeptide), macromomycin. 1,4-benzoquinone derivatives and trenimon.

Toxins are useful as active moieties. When a toxin is conjugated to an ST receptor binding moiety, the conjugated composition is specifically delivered to a metastasized colorectal cell by way of the ST receptor binding moiety and the toxin moiety kills the cell. Toxins are generally complex toxic products various organisms including bacteria, plants, etc. Examples of toxins include but are not limited to: ricin, ricin A chain (ricin toxin), Pseudomonas exotoxin (PE), diphtheria toxin (DT), *Clostridium perfringens* phospholipase C (PLC), bovine pancreatic ribonucleasc (BPR), pokeweed antiviral protein (PAP), abrin, abrin A chain (abrin toxin), cobra venom factor (CVF), gelonin (GEL), saporin (SAP), modeccin, viscumin and volkensin. As discussed above, when protein toxins are employed with ST receptor binding peptides, conjugated compositions may bc produced using recombinant DNA techniques. Briefly, a recombinant DNA molecule can be constructed which encodes both the ST receptor ligand and the toxin on a chimeric gene. When the chimeric gene is expressed, a fusion protein is produced which includes an ST receptor binding moiety and an active moiety. Protein toxins are also useful to form conjugated compounds with ST receptor binding peptides through non-peptidyl bonds.

In addition, there are other approaches for utilizing active agents for the treatment of cancer. For example, conjugated compositions may be produced which include an ST binding moiety and an active moiety which is an active enzyme. The ST binding moiety specifically localizes the conjugated composition to the tumor cells. An inactive prodrug which can be converted by the enzyme into an active drug is administered to the patient. The prodrug is only converted to an active drug by the enzyme which is localized to the tumor. An example of an enzyme/prodrug pair includes alkaline phosphatase/etoposidephosphate. In such a case, the alkaline phosphatase is conjugated to an ST receptor binding ligand. The conjugated compound is administered and localizes at the metastasized cell. Upon contact with etoposidephosphate (the prodrug), the etoposidephosphate is converted to etoposide, a chemotherapeutic drug which is taken up by the cancer cell.

Radiosensitizing agents are substances that increase the sensitivity of cells to radiation. Examplcs of radiosensitizing agents include nitroimidazoles, metronidazole and misonidazole (see: DeVita, V. T. Jr. in *Harrison's Principles of Internal Medicine*, p.68, McGraw-Hill Book Co., New York 1983, which is incorporated herein by reference). The conjugated compound that comprises a radiosensitizing agent as the active moiety is administered and localizes at the metastasized cell. Upon exposure of the individual to radiation, the radiosensitizing agent is "excited" and causes the death of the cell.

Radionuclides may be used in pharmaceutical compositions that are useful for radiotherapy or imaging procedures.

Examples of radionuclides useful as toxins in radiation therapy include: ^{47}Sc, ^{67}Cu, ^{90}Y, ^{109}Pd, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, ^{199}Au, ^{211}At, ^{212}Pb and ^{212}B. Other radionuclides which have been used by those having ordinary skill in the art include: ^{32}P and ^{33}P, ^{71}Gc, ^{77}As, ^{103}Pb, ^{105}Rh, ^{111}Ag, ^{119}Sb, ^{121}Sn, ^{131}Cs, ^{143}Pr, ^{161}Tb, ^{177}Lu, ^{191}Os, ^{193M}Pt, ^{197}Hg, all beta negative and/or auger emitters. Some preferred radionuclides include: ^{90}Y, ^{131}I ^{211}At and ^{212}Pb/^{212}Bi.

According to the present invention, the active moieties may be an imaging agent. Imaging agents are useful diagnostic procedures as well as the procedures used to identify the location of mctastasized cells. Imaging can be performed by many procedures well-known to those having ordinary skill in the art and the appropriate imaging agent useful in such procedures may be conjugated to an ST receptor ligand by well-known means. Imaging can be performed, for example, by radioscintigraphy, nuclear magnetic resonance imaging (MRI) or computed tomography (CT scan). The most commonly employed radionuclide imaging agents include radioactive iodine and indium. Imaging by CT scan may employ a heavy metal such as iron chelates. MRI scanning may employ chelates of gadolinium or manganese. Additionally, positron emission tomography (PET) may be possible using positron emitters of oxygen, nitrogen, iron, carbon, or gallium. Example of radionuclides useful in imaging procedures include: ^{43}K, ^{52}Fe, ^{57}Co, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{77}Br, $^{81}Rb/^{81M}Kr$, ^{87M}Sr, ^{99M}Tc, ^{111}In, ^{113M}In, ^{123}I, ^{125}I, ^{127}Cs, ^{129}Cs, ^{131}I, ^{132}I, ^{197}Hg, ^{203}Pb and ^{206}Bi.

It is preferred that the conjugated compositions be non-immunogenic or immunogenic at a very low level. Accordingly, it is preferred that the ST receptor binding moiety be a small, poorly immunogenic or non-immunogenic peptide or a non-peptide. Likewise, it is preferred that the active moiety be a small, poorly-immunogenic or non-immunogenic peptide or a non-peptide. Native ST, being a small peptide, has been shown to poorly immunogenic. (See: Klipstein, F. A. et al. (1982) *Infect. Immun.* 37:550–557; Giannella, R. A. et al. (1981) *Infect. Immun.* 33:186; Bur-

gess, M. N. et al. (1978) *Infect. Immun.* 21:60; Evans, D. G. et al. (1973) *Infect. Immun.* 7:873; Cyles, C. L. (1979) *Ann. N.Y. Acad. Sci.* 16:314; and Sack, R. B. (1975) *Ann. Rev. Microbiol.* 29:333.) Similarly, fragments and amino acid substitute derivatives of native ST are poorly immunogenic. Accordingly, conjugated compositions which include all or part of the native ST as an ST receptor binding moiety are generally poorly immunogenic to the extent that the native ST is poorly immunogenic.

ST receptor ligands are conjugated to active agents by a variety of well-known techniques readily performed without undue experimentation by those having ordinary skill in the art. The technique used to conjugate the ST receptor ligand to the active agent is dependent upon the molecular nature of the ST receptor ligand and the active agent. After the ST receptor ligand and the active agent are conjugated to form a single molecule, assays may be performed to ensure that the conjugated molecule retains the activities of the moieties. The ST receptor binding assay described above may be performed using the conjugated compound to test whether it is capable of binding to the ST receptor. Similarly, the activity of the active moiety may be tested using various assays for each respective type of active agent. Radionuclides retain there activity, i.e. their radioactivity, irrespective of conjugation. With respect to active agents which are toxins, drugs and targeting agents, standard assays to demonstrate the activity of unconjugated forms of these compounds may be used to confirm that the activity has been retained.

Conjugation may be accomplished directly between the ST receptor ligand and the active agent or linking, intermediate molecular groups may be provided between the ST receptor ligand and the active agent. Crosslinkers are particularly useful to facilitate conjugation by providing attachment sites for each moicty. Crosslinkers may include additional molecular groups which serve as spacers to separate the moieties from each other to prevent either from interfering with the activity of the other.

In some preferred embodiments, the ST receptor ligand peptide is SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5–54 or fragments or derivatives thereof. It has been observed that conjugation to these molecules is preferably performed at the amino terminus of each respective peptide. In ST receptor ligand peptides comprising D amino acid sequences in the opposite order as SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5–54, conjugation preferably is performed at the carboxy terminus.

One having ordinary skill in the art may conjugate an ST receptor ligand peptide to a chemotherapeutic drug using well-known techniques. For example, Magerstadt, M. *Antibody Conjugates and Malignant Disease*. (1991) CRC Press, Boca Raton, USA, pp. 110–152) which is incorporated herein by reference, teaches the conjugation of various cytostatic drugs to amino acids of antibodies. Such reactions may be applied to conjugate chemotherapeutic drugs to ST receptor ligands, including ST receptor binding peptides, with an appropriate linker. ST receptor ligands which have a free amino group such as ST receptor binding peptides may be conjugated to active agents at that group. Most of the chemotherapeutic agents currently in use in treating cancer possess functional groups that are amenable to chemical crosslinking directly with proteins. For example, free amino groups are available on methotrexate, doxorubicin, daunorubicin, cytosinarabinoside, cis-platin, vindesine, mitomycin and bleomycin while free carboxylic acid groups are available on methotrexate, melphalan, and chlorambucil. These functional groups, that is free amino and carboxylic acids, are targets for a variety of homobifunctional and heterobifunctional chemical crosslinking agents which can crosslink these drugs directly to the single free amino group of ST. For example, one procedure for crosslinking ST receptor ligands which have a free amino group such as ST receptor binding peptides, as for example SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5–54 to active agents which have a free amino group such as methotrexate, doxorubicin, daunorubicin, cytosinarabinoside, cis-platin, vindesine, mitomycin and bleomycin, or alkaline phosphatase, or protein- or peptide-based toxin employs homobifunctional succinimidyl esters, preferably with carbon chain spacers such as disuccinimidyl suberate (Pierce Co, Rockford, Ill.). In the event that a clearable conjugated compound is required, the same protocol would be employed utilizing 3,3'-dithiobis (sulfosuccinimidylpropionate; Pierce Co.).

In order to conjugate an ST receptor ligand peptide to a peptide-based active agent such as a toxin, the ST receptor ligand and the toxin may be produced as a single, fusion protein either by standard peptide synthesis or recombinant DNA technology, both of which can be routinely performed by those having ordinary skill in the art. Alternatively, two peptides, the ST receptor ligand peptide and the peptide-based toxin may be produced and/or isolated as separate peptides and conjugated using crosslinkers. As with conjugated compositions that contain chemotherapeutic drugs, conjugation of ST receptor binding peptides and toxins can exploit the ability to modify the single free amino group of an ST receptor binding peptide while preserving the receptor-binding function of this molecule.

One having ordinary skill in the art may conjugate an ST receptor ligand peptide to a radionuclide using well-known techniques. For example, Magerstadt, M. (1991) *Antibody Conjugates And Malignant Disease*, CRC Press, Boca Raton, Fla.,; and Barchel, S. W. and Rhodes, B. H., (1983) *Radioimaging and Radiotherapy*, Elsevier, NY, N.Y., each of which is incorporated herein by reference, teach the conjugation of various therapeutic and diagnostic radionuclides to amino acids of antibodies. Such reactions may be applied to conjugate radionuclides to ST receptor ligand peptides or to ST receptor ligands including ST receptor ligand peptides with an appropriate linker.

The present invention provides pharmaceutical compositions that comprise the conjugated compounds of the invention and pharmaceutically acceptable carriers or diluents. The pharmaceutical composition of the present invention may be formulated by one having ordinary skill in the art. Suitable pharmaceutical carriers are described in *Remington's Pharmaceutical Sciences*, A. Osol, a standard reference text in this field, which is incorporated herein by reference. In carrying out methods of the present invention, conjugated compounds of the present invention can be used alone or in combination with other diagnostic, therapeutic or additional agents. Such additional agents include excipients such as coloring, stabilizing agents, osmotic agents and antibacterial agents.

The conjugated compositions of the invention can be, for example, formulated as a solution, suspension or emulsion in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes may also be used. The vehicle may contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). The formulation is sterilized by commonly used techniques. For example, a parenteral composition suitable for administration by injection is prepared by dissolving

1.5% by weight of active ingredient in 0.9% sodium chloride solution.

The pharmaceutical compositions according to the present invention may be administered as either a single dose or in multiple doses. The pharmaceutical compositions of the present invention may be administered either as individual therapeutic agents or in combination with other therapeutic agents. The treatments of the present invention may be combined with conventional therapies, which may be administered sequentially or simultaneously.

The pharmaceutical compositions of the present invention may be administered by any means that enables the conjugated composition to reach the targeted cells. In some embodiments, routes of administration include those selected from the group consisting of intravenous, intraarterial, intraperitoneal, local administration into the blood supply of the organ in which the tumor resides or directly into the tumor itself. Intravenous administration is the preferred mode of administration. It may be accomplished with the aid of an infusion pump.

The dosage administered varies depending upon factors such as: the nature of the active moiety; the nature of the conjugated composition; pharmacodynamic characteristics; its mode and route of administration; age, health, and weight of the recipient; nature and extent of symptoms; kind of concurrent treatment; and frequency of treatment.

Because conjugated compounds are specifically targeted to cells with ST receptors, conjugated compounds which comprise chemotherapeutics or toxins are administered in doses less than those which are used when the chemotherapeutics or toxins are administered as unconjugated active agents, preferably in doses that contain up to 100 times less active agent. In some embodiments, conjugated compounds which comprise chemotherapeutics or toxins are administered in doses that contain 10–100 times less active agent as an active moiety than the dosage of chemotherapeutics or toxins administered as unconjugated active agents. To determine the appropriate dose, the amount of compound is preferably measured in moles instead of by weight. In that way, the variable weight of different ST binding moieties does not affect the calculation. Presuming a one to one ratio of ST binding moiety to active moiety in conjugated compositions of the invention, less moles of conjugated compounds may be administered as compared to the moles of unconjugated compounds administered, preferably up to 100 times less molcs.

Typically, chemotherapeutic conjugates are administered intravenously in multiple divided doses.

Up to 20 gm IV/dose of methotrexate is typically administered in an unconjugated form. When methotrexate is administered as the active moiety in a conjugated compound of the invention, there is a 10- to 100-fold dose reduction. Thus, presuming each conjugated compound includes one molecule of methotrexate conjugated to one ST receptor binding moiety, of the total amount of conjugated compound administered, up to about 0.2–2.0 g of methotrexate is present and therefore administered. In some embodiments, of the total amount of conjugated compound administered, up to about 200 mg–2 g of methotrexate is present and therefore administered.

Methotrexate has a molecular weight of 455. One mole of the ST peptide-methotrexate conjugate weighs between about 1755–2955 depending on the ST peptide used. The effective dose range for ST peptide-methotrexate conjugate is between about 10 to 1000 mg. In some embodiments, dosages of 50 to 500 mg of ST peptide-methotrexate conjugate are administered. In some embodiments, dosages of 80 to 240 mg of ST peptide-methotrexate conjugate are administered.

Doxorubicin and daunorubicin each weigh about 535. Thus, ST peptide-doxorubicin conjugates and ST peptide-daunorubicin conjugates each have molecular weights of between about 1835–2553.5. Presuming each conjugated compound includes one molecule of doxorubicin or daunorubicin conjugated to one ST receptor binding moiety, the effective dose range for ST peptide-doxorubicin conjugate or ST peptide-daunorubicin conjugate is between about 40 to 4000 mg. In some embodiments, dosages of 100 to 1000 mg of ST peptide-doxorubicin conjugate or ST peptide-daunorubicin conjugate are administered. In some embodiments, dosages of 200 to 600 mg of ST peptide-doxorubicin conjugate or ST peptide-daunorubicin conjugate are administered.

Toxin-containing conjugated compounds are formulated for intravenous administration. Using this approach, up to 6 nanomoles/kg of body weight of toxin have been administered as a single dose with marked therapeutic effects in patients with melanoma (Spitler L. E., et al. (1987) *Cancer Res.* 47:1717). In some embodiments, up to about 11 micrograms of ST peptide-toxin conjugated compound/kg of body weight may be administered for therapy.

Presuming each conjugated compound includes one molecule of ricin toxin A chain conjugated to an ST receptor binding moiety, conjugated compositions comprising ricin toxin A chain are administered in doses in which the proportion by weight of ricin toxin A chain is 1–500 μg of the total weight of the conjugated compound administered. In some preferred embodiments, conjugated compositions comprising ricin toxin A chain are administered in doses in which the proportion by weight of ricin toxin A chain is 10–100 μg of the total weight of the conjugated compound administered. In some preferred embodiments, conjugated compositions comprising ricin toxin A chain are administered in doses in which the proportion by weight of ricin toxin A chain is 2–50 μg of the total weight of the conjugated compound administered. The molecular weight of ricin toxin A chain is 32,000. Thus, a conjugated compound that contains ricin A chain linked to an ST peptide has a molecular weight of about 33,300–34,500. The range of doses of such conjugated compounds to be administered are 1 to 500 μg. In some embodiments, 10 to 100 μg of such conjugated compounds are administered. In some embodiments, 20 to 50 μg of such conjugated compounds are administered.

Presuming each conjugated compound includes one molecule of diphtheria toxin A chain conjugated to an ST receptor binding moiety, conjugated compositions comprising diphtheria toxin A chain are administered in doses in which the proportion by weight of diphtheria toxin A chain is 1–500 μg of the total weight of the conjugated compound administered. In some preferred embodiments, conjugated compositions comprising diphtheria toxin A chain are administered in doses in which the proportion by weight of diphtheria toxin A chain is 10–100 μg of the total weight of the conjugated compound administered. In some preferred embodiments, conjugated compositions comprising diphtheria toxin A chain are administered in doses in which the proportion by weight of diphtheria toxin A chain is 40–80 μg of the total weight of the conjugated compound administered. The molecular weight of diphtheria toxin A chain is 66,600. Thus, a conjugated compound that contains diphtheria A chain linked to an ST peptide has a molecular weight of about 67,900–69,100. The range of doses of such

conjugated compounds to be administered tested are 1 to 500 μg. In some embodiments, 10 to 100 μg of such conjugated compounds are administered. In some embodiments, 40 to 80 μg of such conjugated compounds are administered.

Presuming each conjugated compound includes one molecule of Pseudomonas exotoxin conjugated to an ST receptor binding moiety, conjugated compositions comprising Pseudomonas exotoxin are administered in doses in which the proportion by weight of Pseudomonas exotoxin is 0.01–100 μg of the total weight of the conjugated compound administered. In some preferred embodiments, conjugated compositions comprising Pseudomonas exotoxin are administered in doses in which the proportion by weight of Pseudomonas exotoxin is 0.1–10 μg of the total weight of the conjugated compound administered. In some preferred embodiments, conjugated compositions comprising Pseudomonas exotoxin are administered in doses in which the proportion by weight of Pseudomonas exotoxin is 0.3–2.2 μg of the total weight of the conjugated compound administered. The molecular weight of Pseudomonas exotoxin is 22,000. Thus, a conjugated compound that contains Pseudomonas exotoxin linked to an ST peptide has a molecular weight of about 23,300–24,500. The range of doses of such conjugated compounds to be administered tested are 0.01 to 100 μg. In some embodiments, 0.1 to 10 μg of such conjugated compounds are administered. In some embodiments, 0.3 to 2.2 μg of such conjugated compounds are administered.

To dose conjugated compositions comprising ST receptor binding moieties linked to active moieties that are radioisotopes in pharmaceutical compositions useful as imaging agents, it is presumed that each ST receptor binding moiety is linked to one radioactive active moiety. The amount of radioisotope to be administered is dependent upon the radioisotope. Those having ordinary skill in the art can readily formulate the amount of conjugated compound to be administered based upon the specific activity and energy of a given radionuclide used as an active moiety. Typically 0.1–100 millicuries per dose of imaging agent, preferably 1–10 millicuries, most often 2–5 millicuries are administered. Thus, pharmaceutical compositions according to the present invention useful as imaging agents which comprise conjugated compositions comprising an ST receptor binding moiety and a radioactive moiety comprise 0.1–100 millicuries, in some embodiments preferably 1–10 millicuries, in some embodiments preferably 2–5 millicuries, in some embodiments more preferably 1–5 millicuries. Examples of dosages include: ^{131}I=between about 0.1–100 millicuries per dose, in some embodiments preferably 1–10 millicuries, in some embodiments 2–5 millicuries, and in some embodiments about 4 millicuries; ^{111}In=between about 0.1–100 millicuries per dose, in some embodiments preferably 1–10 millicuries, in some embodiments 1–5 millicuries, and in some embodiments about 2 millicuries; ^{99m}Tc=between about 0.1–100 millicuries per dose, in some embodiments preferably 5–75 millicuries, in some embodiments 10–50 millicuries, and in some embodiments about 27 millicuries. Depending upon the specific activity of the radioactive moiety and the weight of the ST receptor binding moiety the dosage defined by weight varies. ST peptides have molecular weights of between about 1300–2500. In the pharmaceutical composition comprising an ST peptide linked to a single ^{131}I in which the specific activity of ^{131}I-ST peptide is about 2000 Ci/mmol, administering the dose of 0.1–100 millicuries is the equivalent of 0.1–100 μg ^{131}I-ST peptide, administering the dose of 1–10 millicuries is the equivalent of 1–10 μg of ^{131}I-ST peptide, administering the dose of 2–5 millicuries is equivalent to giving 2–5 μg of ^{131}I-ST peptide and administering the dose of 1–5 millicuries is equivalent to giving 1–5 μg of ^{131}I-ST peptide. In the pharmaceutical composition comprising an ST peptide linked to a single ^{111}In in which the specific activity of ^{111}In-ST peptide is about 1 Ci/mmol, administering the dose of 0.1–100 millicuries is the equivalent of 0.2–200 mg ^{111}In-ST peptide, administering the dose of 1–10 millicuries is the equivalent of 2–20 mg of ^{111}In-ST peptide, administering the dose of 2–5 millicuries is equivalent to giving 4–10 mg of ^{111}In-ST peptide and administering the dose of 1–5 millicuries is equivalent to giving 2–10 mg of ^{111}In-ST peptide.

To dose conjugated compositions comprising ST receptor binding moieties linked to active moieties that are radioisotopes in pharmaceutical compositions useful as therapeutic agents, it is presumed that each ST receptor binding moiety is linked to one radioactive active moiety. The amount of radioisotope to be administered is dependent upon the radioisotope. Those having ordinary skill in the art can readily formulate the amount of conjugated compound to be administered based upon the specific activity and energy of a given radionuclide used as an active moiety. For therapeutics that comprise ^{131}I, between 10–1000 mM, preferably 50–500, more preferably about 300 nanomoles of ^{131}I at the tumor, per gram of tumor, is desirable. Thus, if there is about 1 gram of tumor, and about 0.1% of the administered dose binds to the tumor, 0.5–100 mg of ^{131}I-ST peptide conjugated compound is administered. In some embodiments, 1 to 50 mg of ^{131}I-ST peptide conjugated compound is administered. In some embodiments, 5 to 10 mg of ^{131}I-ST peptide conjugated compound is administered. Wessels B. W. and R. D. Rogus (1984) *Med. Phys.* 11:638 and Kwok, C. S. et al. (1985) *Med. Phys.* 12:405, both of which are incorporated herien by reference, disclose detailed dose calculations for diagnostic and therapeutic conjugates which may be used in the preparation of pharmaceutical compositions of the present invention which include radioactive conjugated compounds.

One aspect of the present invention relates to a method of treating individuals suspected of suffering from metastasized colorectal cancer. Such individuals may be treated by administering to the individual a pharmaceutical composition that comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radiostable therapeutic agent. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radiostable active agent and the ST receptor binding moiety is a peptide. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radiostable active agent and the ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5–54 and fragments and derivatives thereof. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radiostable active agent and the ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ

ID NO:3, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:54. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radiostable agent. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radiostable agent selected from the group consisting of: methotrexate, doxorubicin, daunorubicin, cytosinarabinoside, etoposide, 5-4 fluorouracil. melphalan, chlorambucil, cis-platinum, vindesine, mitomycin, bleomycin, purothionin, macromomycin, 1,4 -benzoquinone derivatives, trenimon, ricin, ricin A chain, Pseudomonas cxotoxin, diphtheria toxin, *Clostridium perfringens* phospholipase C, bovine pancreatic ribonuclease, pokeweed antiviral protein, abrin, abrin A chain, cobra venom factor, gelonin, saporin, modeccin, viscumin, volkensin, alkaline phosphatase, nitroimidazole, metronidazole and misonidazole. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5–54 and fragments and derivatives thereof and the active moiety is a radiostable active agent selected from the group consisting of: methotrexate, doxorubicin, daunorubicin, cytosinarabinoside, etoposide, 5-4 fluorouracil, melphalan, chlorambucil, cis-platinum, vindesine, mitomycin, bleomycin, purothionin, macromomycin, 1,4-benzoquinone derivatives, trenimon, ricin, ricin A chain, Pseudomonas exotoxin, diphtheria toxin, *Clostridium perfringens* phospholipase C, bovine pancreatic ribonuclcasc, pokeweed antiviral protein, abrin, abrin A chain, cobra venom factor, gelonin, saporin, modeccin, viscumin, volkensin, alkaline phosphatase, nitroimidazole, metronidazole and misonidazole. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radiostable active agent selected from the group consisting of: mcthotrcxatc, doxorubicin, daunorubicin, cytosinarabinoside, cis-platin, vindesine, mitomycin and bleomycin, alkaline phosphatase, ricin A chain, Pseudomonas exotoxin and diphtheria toxin. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:54 and the active moiety is a radiostable active agent selected from the group consisting of: methotrexate, doxorubicin, daunorubicin, cytosinarabinoside, cis-platin, vindesine, mitomycin and bleomycin, alkaline phosphatase, ricin A chain, Pseudomonas exotoxin and diphtheria toxin. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a radiostable conjugated compound described in Example 1. The individual being treated may be diagnosed as having metastasized colorectal cancer or may be diagnosed as having localized colorectal cancer and may undergo the treatment proactively in the event that there is some metastasis as yet undetected. The pharmaceutical composition contains a therapeutically effective amount of the conjugated composition. A therapeutically effective amount is an amount which is effective to cause a cytotoxic or cytostatic effect on metastasized colorectal cancer cells without causing lethal side effects on the individual.

One aspect of the present invention relates to a method of treating individuals suspected of suffering from metastasized colorectal cancer. Such individuals may be treated by administering to the individual a pharmaceutical composition that comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radioactive. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radioactive and the ST receptor binding moiety is a peptide. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radioactive and the ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5–54 and fragments and derivativcs thercof. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radioactive and the ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5, SEQ ID NO:6 and SEQ ID NO:54. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the activc moiety is a radioactive agent selected from the group consisting of: ^{47}Sc, ^{67}Cu, ^{90}Y, ^{109}Pd, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, ^{199}Au, ^{211}At, ^{212}Pb, ^{212}B, ^{32}P and ^{33}P, ^{71}Ge, ^{77}As, ^{103}Pb, ^{105}Rh, ^{111}Ag, ^{119}Sb, ^{121}Sn, ^{131}Cs, ^{143}Pr, ^{161}Tb, ^{177}Lu, ^{191}Os, ^{193m}Pt, ^{197}Hg. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5–54 and fragments and derivatives thereof and the active moiety is a radioactive agent selected from the group consisting of: ^{47}Sc, ^{67}Cu, ^{90}Y, ^{109}Pd, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, ^{199}Au, ^{211}At, ^{212}Pb, ^{212}B, ^{32}P and ^{33}P, ^{71}Ge, ^{77}As, ^{103}Pb, ^{105}Rh, ^{111}Ag, ^{119}Sb, ^{121}Sn, ^{131}Cs, ^{143}Pr, ^{161}Tb, ^{177}Lu, ^{191}Os, ^{193m}Pt, ^{197}Hg, all beta negative and/or auger emitters. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radioactive agent selected from the group consisting of: ^{47}Sc, ^{67}Cu, ^{90}Y, ^{109}Pd, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, ^{199}Au, ^{211}At, ^{212}Pb, ^{212}B, ^{32}P and ^{33}P, ^{71}Ge, ^{77}As, ^{103}Pb, ^{105}Rh, ^{111}Ag, ^{119}Sb, ^{121}Sn, ^{131}Cs, ^{143}Pr, ^{161}Tb, ^{177}Lu, ^{191}Os, ^{193m}Pt, ^{197}Hg. In some

embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:54 and the active moiety is a radioactive agent selected from the group consisting of: ^{47}Sc, ^{67}Cu, ^{90}Y, ^{109}Pd, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, ^{199}Au, ^{211}At, ^{212}Pb, ^{212}B, ^{32}P and ^{33}P, ^{71}Ge, ^{77}As, ^{103}Pb, ^{105}Rh, ^{111}Ag, ^{119}Sb, ^{121}Sn, ^{131}Cs, ^{143}Pr, ^{161}Tb, ^{177}Lu, ^{191}Os, ^{193m}Pt, ^{197}Hg. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a radioactive conjugated compound described in Example 1. The individual being treated may be diagnosed as having metastasized colorectal cancer or may be diagnosed as having localized colorectal cancer and may undergo the treatment proactively in the event that there is some metastasis as yet undetected. The pharmaceutical composition contains a therapeutically effective amount of the conjugated composition. A therapeutically effective amount is an amount which is effective to cause a cytotoxic or cytostatic effect on metastasized colorectal cancer cells without causing lethal side effects on the individual.

One aspect of the present invention relates to a method of detecting metastasized colorectal cancer cells in an individual suspected of suffering from metastasized colorectal cancer by radioimaging. Such individuals may be diagnosed as suffering from metastasized colorectal cancer and the metastasized colorectal cancer cells may be detected by administering to the individual, preferably by intravenous administration, a pharmaceutical composition that comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radioactive and detecting the presence of a localized accumulation or aggregation of radioactivity, indicating the presence of cells with ST receptors. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radioactive and the ST receptor binding moiety is a peptide. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radioactive and the ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5–54 and fragments and derivatives thereof. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radioactive and the ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:54. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radioactive agent selected from the group consisting of: radioactive heavy metals such as iron chelates, radioactive chelates of gadolinium or manganese, positron emitters of oxygen, nitrogen, iron, carbon, or gallium, ^{43}K, ^{52}Fe, ^{57}Co, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{77}Br, $^{81}Rb/^{81m}Kr$, ^{87m}Sr, ^{99m}Tc, ^{111}In, ^{113m}In, ^{123}I, ^{125}I, ^{127}Cs, ^{129}Cs, ^{131}I, ^{132}I, ^{197}Hg, ^{203}Pb and ^{206}Bi. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5-54 and fragments and derivatives thereof and the active moiety is a radioactive agent selected from the group consisting of: radioactive heavy metals such as iron chelates, radioactive chelates of gadolinium or manganese, positron emitters of oxygen, nitrogen, iron, carbon, or gallium, ^{43}K, ^{52}Fe, ^{57}Co, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{77}Br, $^{81}Rb/^{81m}Kr$, ^{87m}Sr, ^{99m}Tc, ^{111}In, ^{113m}In, ^{123}I, ^{125}I, ^{127}Cs, ^{129}Cs, ^{131}I, ^{132}I, ^{197}Hg, ^{203}Pb and ^{206}Bi. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is a radioactive agent selected from the group consisting of: ^{43}K, ^{52}Fe, ^{57}Co, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{77}Br, $^{81}Rb/^{81m}Kr$, ^{87m}Sr, ^{99m}Tc, ^{111}In, ^{113m}In, ^{123}I, ^{125}I, ^{127}Cs, ^{129}Cs, ^{131}I, ^{132}I, ^{197}Hg, ^{203}Pb and ^{206}Bi. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a conjugated compound that comprises an ST receptor binding moiety and an active moiety wherein the active moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:54 and the active moiety is a radioactive agent selected from the group consisting of: ^{43}K, ^{52}Fe, ^{57}Co, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{77}Br, $^{81}Rb/^{81m}Kr$, ^{87m}Sr, ^{99m}Tc, ^{111}In, ^{113m}In, ^{123}I, ^{125}I, ^{127}Cs, ^{129}Cs, ^{131}I, ^{132}I, ^{197}Hg, ^{203}Pb and ^{206}Bi. In some embodiments of the present invention, the pharmaceutical composition comprises a pharmaceutically acceptable carrier or diluent and a radioactive conjugated compound described in Example 1. The individual being treated may be diagnosed as having metastasized colorectal cancer or may be diagnosed as having localized colorectal cancer and may undergo the treatment proactively in the event that there is some metastasis as yet undetected. The pharmaceutical composition contains a diagnostically effective amount of the conjugated composition. A diagnostically effective amount is an amount which can be detected at a site in the body where cells with ST receptors are located without causing lethal side effects on the individual.

Another aspect of the invention relates to unconjugated compositions which comprise an ST receptor binding ligand and an active agent. For example, liposomes are small vesicles composed of lipids. Drugs can be introduced into the center of these vesicles. The outer shell of these vesicles comprise an ST receptor binding ligand. *Liposomes* Volumes 1, 2 and 3 CRC Press Inc. Boca Raton, Fla., which is incorporated herein by reference, disclose preparation of liposome-encapsulated active agents which include targeting agents that correspond to ST receptor ligand in the outer shell. Unconjugated compositions which comprise an ST receptor ligand in the matrix of a liposome with an active agent inside include such compostions in which the ST receptor ligand is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5–54 and fragments and derivatives thereof and the active agent is selected from the group consisting of: methotrexate, doxorubicin, daunorubicin, cytosinarabinoside, etoposide, 5-fluorouracil, melphalan, chlorambucil, cis-platinum, vin-

desine, mitomycin, bleomycin, purothionin, macromomycin, 1,4 -benzoquinone derivatives, trenimon, ricin, ricin A chain, Pseudomonas exotoxin, diphtheria toxin, *Clostridium perfringens* phospholipase C, hovinc pancreatic ribonuclease, pokeweed antiviral protein, abrin, abrin A chain, cobra venom factor, gelonin, saporin, modeccin, viscumin, volkensin, alkaline phosphatase, nitroimidazole, metronidazole and misonidazole.

Another aspect of the invention relates to unconjugated and conjugated compositions which comprise an ST receptor ligand used to deliver therapeutic nucleic acid molecules to cells that comprise an ST receptor such as normal cells of the intestinal tract as well as metastasized colorectal cancer cells. In some embodiments, the genetic material is delivered to metastasized tumor cells to produce an antigen that can be targeted by the immune system or to produce a protein which kills the cell or inhibits its proliferation. In some embodiments, the ST receptor ligand is used to deliver nucleic acids that encode nucleic acid molecules which replace defective endogenous genes or which encode therapeutic proteins. In some embodiments, the ST receptor ligand is thus used to deliver the active agent specifically to the cells lining the intestinal tract to treat diseases specific to this organ. According to this aspect of the invention, compositions comprise nucleic acid molecules which can replace defective genes. In some embodiments, the compositions are used in gene therapy protocols to deliver to individuals, genetic material needed and/or desired to make up for a genetic deficiency.

In some embodiments, the ST receptor ligand is combined with or incorporated into a delivery vehicle thereby converting the delivery vehicle into a specifically targeted delivery vehicle. For example, an ST receptor binding peptide may be integrated into the outer portion of a viral particle making such a virus an ST receptor-bearing cell specific virus. Similarly, the coat protein of a virus may be engineered such that it is produced as a fusion protein which includes an active ST receptor binding peptide that is exposed or otherwise accessible on the outside of the viral particle making such a virus an ST receptor-bearing cell-specific virus. In some embodiments, an ST receptor ligand may be integrated or otherwise incorporated into the liposomes wherein the ST receptor ligand is exposed or otherwise accessible on the outside of the liposome making such liposomes specifically targeted to ST receptor-bearing cells.

The active agent in the conjugated or unconjugated compositions according to this aspect of the invention is a nucleic acid molecule. The nucleic acid may be RNA or preferably DNA. In some embodiments, the nucleic acid molecule is an antisense molecule or encodes an antisense sequence whose presence in the cell inhibits production of an undesirable protein. In some embodiments, the nucleic acid molecule encodes a ribozyme whose presence in the cell inhibits production of an undesirable protein. In some embodiments, the nucleic acid molecule encodes a protein or peptide that is desirably produced in the cell. In some embodiments, the nucleic acid molecule encodes a functional copy of a gene that is defective in the targeted cell. The nucleic acid molecule is preferably operably linked to regulatory elements needed to express the coding sequence in the cell.

Liposomes are small vesicles composed of lipids. Genetic constructs which encode proteins that are desired to be expressed in ST receptor-bearing cells are introduced into the center of these vesicles. The outer shell of these vesicles comprise an ST receptor ligand, in some embodiments preferably an ST peptide. *Liposomes* Volumes 1, 2 and 3 CRC Press Inc. Boca Raton, Fla., which is incorporated herein by reference, disclose preparation of liposome-encapsulated active agents which include antibodies in the outer shell. In the present invention, an ST receptor ligand such as for example an ST peptide corresponds to the antibodies in the outer shell. Unconjugated compositions which comprise an ST receptor ligand in the matrix of a liposome with an active agent inside include such compositions in which the ST receptor ligand is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5–54 and fragments and derivatives thereof.

In one embodiment for example, cystic fibrosis, a genetic disease in which there is a mutation of a specific gene encoding a chloride transport protein which ultimately produces abnormalities of function in many systems, most notably in the respiratory and intestinal tract, is treated by gene therapy techniques using ST receptor ligands to deliver the corrective gene to cells. Current therapy has been directed at replacing the mutant gene in the respiratory system with the normal gene by targeting these genes directly to the cells lining the respiratory tract using viruses which bind only to those cells. Similarly, the normal gene is packaged in liposomes targeted on their surface with ST receptor ligands and delivered to the intestinal tract. ST receptor ligands specifically target and direct the liposomes containing the normal gene to correct the lesion for cystic fibrosis to the specific cells lining the intestinal tract, from the duodenum to the rectum. Uptake of that genetic material by those cells should result in a cure of cystic fibrosis in the intestinal tract.

In another embodiment, the delivery of normal copies of the p53 tumor suppressor gene to the intestinal tract is accomplished using ST receptor ligand to target the gene therapeutic. Mutations of the p53 tumor suppressor gene appears to play a prominent role in the development of colorectal cancer in the intestinal tract. One approach to combatting this disease is the delivery of normal copies of this gene to the intestinal tract to cells expressing mutant forms of this gene. Genetic constructs that comprise normal p53 tumor suppressor genes are incorporated into liposomes that comprise an ST receptor ligand. The composition is delivered to the intestinal tract. ST receptor binding ligands specifically target and direct the liposomes containing the normal gene to correct the lesion created by mutation of p53 suppressor gene in intestinal cells.

Preparation of genetic constructs is with the skill of those having ordinary skill in the art. The present invention allows such construct to be specifically targeted by using the ST receptor ligands of the present invention. The compositions of the invention include an ST receptor ligand such as an ST peptide associated with a delivery vehicle and a gene construct which comprises a coding sequence for a protein whose production is desired in the cells of the intestinal tract linked to necessary regulatory sequences for expression in the cells. For uptake by cells of the intestinal tract, the compositions are administered orally or by enema whereby they enter the intestinal tract and contact cells which comprise ST receptors. The delivery vehicles associate with the ST receptor by virtue of the ST receptor ligand and the vehicle is internalized into the cell or the active agent/genetic construct is otherwise taken up by the cell. Once internalized, the construct can provide a therapeutic effect on the individual. One having ordinary skill in the art can readily formulate such compositions for oral or enema administration and determine the effective amount of such composition to be administered to treat the disease or disorder.

The following examples are illustrative but are not meant to be limiting of the present invention.

EXAMPLES

Example 1

The following are representative compounds according to the present invention. Whenever stated below, reference to a series of compounds is provided for efficiency and is meant to name each compound in the series including all the compounds in numerical order, such as for example "3-D1 to 3-D16" is meant to refer to compounds 3-D1, 3-D2, 3-D3, 3-D4, 3-D5, 3-D6, 3-D7, 3-D8, 3-D9, 3-D10, 3-D11, 3-D12, 3-D13, 3-D14, 3-D15 and 3-D16. Likewise, whenever stated below, reference to a series of SEQ ID NO.'s is provided for efficiency and is meant to name each SEQ ID NO: in the series including the all SEQ ID NO:'s in numerical order, such as for example SEQ ID NO:5 through SEQ ID NO:54 is meant to refer to SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53 and SEQ ID NO:54. Similarly, whenever stated below, reference to a series of compounds is provided for efficiency and is meant to name each compound in the series including the all compounds in numerical order, such as for example "5-AP to 54-AP" is meant to refer to compounds 5-AP, 6-AP, 7-AP, 8-AP, 9-AP, 10-AP, 11-AP, 12-AP, 13-AP, 14-AP, 15-AP, 16-AP, 17-AP, 18-AP, 19-AP, 20-AP, 21-AP, 22-AP, 23-AP, 24-AP, 25-AP, 26-AP, 27-AP, 28-AP, 29-AP, 30-AP, 31-AP, 32-AP, 33-AP, 34-AP, 35-AP, 36-AP, 37-AP, 38-AP, 39-AP, 40-AP, 41-AP, 42-AP, 43-AP, 44-AP, 45-AP, 46-AP, 47-AP, 48-AP, 49-AP, 50-AP, 51-AP, 52-AP, 53-AP and 54-AP.

Compound 2-D1 comprises methotrexate (amethopterin) conjugated to SEQ ID NO:2.

Compound 2-D2 comprises doxorubicin (adrimycin) conjugated to SEQ ID NO:2.

Compound 2-D3 comprises daunorubicin conjugated to SEQ ID NO:2.

Compound 2-D4 comprises cytosinarabinoside conjugated to SEQ ID NO:2.

Compound 2-D5 comprises etoposide conjugated to SEQ ID NO:2.

Compound 2-D6 comprises 5-4 fluorouracil conjugated to SEQ ID NO:2.

Compound 2-D7 comprises melphalan conjugated to SEQ ID NO:2.

Compound 2-D8 comprises chlorambucil conjugated to SEQ ID NO:2.

Compound 2-D9 comprises cyclophosphamide conjugated to SEQ ID NO:2.

Compound 2-D10 comprises cis-platinum conjugated to SEQ ID NO:2.

Compound 2-D11 comprises vindesine conjugated to SEQ ID NO:2.

Compound 2-D12 comprises mitomycin conjugated to SEQ ID NO:2.

Compound 2-D13 comprises bleomycin conjugated to SEQ ID NO:2.

Compound 2-D14 comprises purothionin conjugated to SEQ ID NO:2.

Compound 2-D15 comprises macromomycin conjugated to SEQ ID NO:2.

Compound 2-D16 comprises trenimon conjugated to SEQ ID NO:2.

Compounds 3-D1 to 3-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 3-D1 to 3-D16 each comprise SEQ ID NO:3 as the ST receptor binding moiety.

Compounds 5-D1 to 5-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 5-D1 to 5-D16 each comprise SEQ ID NO:5 as the ST receptor binding moiety.

Compounds 6-D1 to 6-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 6-D1 to 6-D16 each comprise SEQ ID NO:6 as the ST receptor binding moiety.

Compounds 7-D1 to 7-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 7-D1 to 7-D16 each comprise SEQ ID NO:7 as the ST receptor binding moiety.

Compounds 8-D1 to 8-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 8-D1 to 8-D16 each comprise SEQ ID NO:8 as the ST receptor binding moiety.

Compounds 9-D1 to 9-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 9-D1 to 9-D16 each comprise SEQ ID NO:9 as the ST receptor binding moiety.

Compounds 10-D1 to 10-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 10-D1 to 10-D16 each comprise SEQ ID NO:2 as the ST receptor binding moiety.

Compounds 12-D1 to 12-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 12-D1 to 12-D16 each comprise SEQ ID NO:11 as the ST receptor binding moiety.

Compounds 12-D1 to 12-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 12-D1 to 12-D16 each comprise SEQ ID NO:12 as the ST receptor binding moiety.

Compounds 13-D1 to 13-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 13-D1 to 13-D16 each comprise SEQ ID NO:13 as the ST receptor binding moiety.

Compounds 14-D1 to 14-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising

SEQ ID NO:2 as the ST receptor binding moiety, compounds 14-D1 to 14-D16 each comprise SEQ ID NO:14 as the ST receptor binding moiety.

Compounds 15-D1 to 15-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 15-D1 to 15-D16 each comprise SEQ ID NO:15 as the ST receptor binding moiety.

Compounds 16-D1 to 16-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 16-D1 to 16-D16 each comprise SEQ ID NO:16 as the ST receptor binding moiety.

Compounds 17-D1 to 17-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 17-D1 to 17-D16 each comprise SEQ ID NO:17 as the ST receptor binding moiety.

Compounds 18-D1 to 18-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 18-D1 to 18-D16 each comprise SEQ ID NO:18 as the ST receptor binding moiety.

Compounds 19-D1 to 19-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 19-D1 to 19-D16 each comprise SEQ ID NO:19 as the ST receptor binding moiety.

Compounds 20-D1 to 20-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 20-D1 to 20-D16 each comprise SEQ ID NO:20 as the ST receptor binding moiety.

Compounds 22-D1 to 22-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 22-D1 to 22-D16 each comprise SEQ ID NO:21 as the ST receptor binding moiety.

Compounds 22-D1 to 22-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 22-D1 to 22-D16 each comprise SEQ ID NO:22 as the ST receptor binding moiety.

Compounds 23-D1 to 23-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 23-D1 to 23-D16 each comprise SEQ ID NO:23 as the ST receptor binding moiety.

Compounds 24-D1 to 24-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 24-D1 to 24-D16 each comprise SEQ ID NO:24 as the ST receptor binding moiety.

Compounds 25-D1 to 25-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 25-D1 to 25-D16 each comprise SEQ ID NO:25 as the ST receptor binding moiety.

Compounds 26-D1 to 26-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 26-D1 to 26-D16 each comprise SEQ ID NO:26 as the ST receptor binding moiety.

Compounds 27-D1 to 27-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 27-D1 to 27-D16 each comprise SEQ ID NO:27 as the ST receptor binding moiety.

Compounds 28-D1 to 28-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 28-D1 to 28-D16 each comprise SEQ ID NO:28 as the ST receptor binding moiety.

Compounds 29-D1 to 29-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 29-D1 to 29-D16 each comprise SEQ ID NO:29 as the ST receptor binding moiety.

Compounds 30-D1 to 30-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 30-D1 to 30-D16 each comprise SEQ ID NO:30 as the ST receptor binding moiety.

Compounds 32-D1 to 32-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 32-D1 to 32-D16 each comprise SEQ ID NO:31 as the ST receptor binding moiety.

Compounds 32-D1 to 32-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 32-D1 to 32-D16 each comprise SEQ ID NO:32 as the ST receptor binding moiety.

Compounds 33-D1 to 33-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 33-D1 to 33-D16 each comprise SEQ ID NO:33 as the ST receptor binding moiety.

Compounds 34-D1 to 34-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 34-D1 to 34-D16 each comprise SEQ ID NO:34 as the ST receptor binding moiety.

Compounds 35-D1 to 35-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 35-D1 to 35-D16 each comprise SEQ ID NO:35 as the ST receptor binding moiety.

Compounds 36-D1 to 36-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 36-D1 to 36-D16 each comprise SEQ ID NO:36 as the ST receptor binding moiety.

Compounds 37-D1 to 37-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 37-D1 to 37-D16 each comprise SEQ ID NO:37 as the ST receptor binding moiety.

Compounds 38-D1 to 38-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 38-D1 to 38-D16 each comprise SEQ ID NO:38 as the ST receptor binding moiety.

Compounds 39-D1 to 39-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 39-D1 to 39-D16 each comprise SEQ ID NO:39 as the ST receptor binding moiety.

Compounds 40-D1 to 40-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising

SEQ ID NO:2 as the ST receptor binding moiety, compounds 40-D1 to 40-D16 each comprise SEQ ID NO:40 as the ST receptor binding moiety.

Compounds 42-D1 to 42-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 42-D1 to 42-D16 each comprise SEQ ID NO:41 as the ST receptor binding moiety.

Compounds 42-D1 to 42-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 42-D1 to 42-D16 each comprise SEQ ID NO:42 as the ST receptor binding moiety.

Compounds 43-D1 to 43-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 43-D1 to 43-D16 each comprise SEQ ID NO:43 as the ST receptor binding moiety.

Compounds 44-D1 to 44-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 44-D1 to 44-D16 each comprise SEQ ID NO:44 as the ST receptor binding moiety.

Compounds 45-D1 to 45-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 45-D1 to 45-D16 each comprise SEQ ID NO:45 as the ST receptor binding moiety.

Compounds 46-D1 to 46-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 46-D1 to 46-D16 each comprise SEQ ID NO:46 as the ST receptor binding moiety.

Compounds 47-D1 to 47-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 47-D1 to 47-D16 each comprise SEQ ID NO:47 as the ST receptor binding moiety.

Compounds 48-D1 to 48-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 48-D1 to 48-D16 each comprise SEQ ID NO:48 as the ST receptor binding moiety.

Compounds 49-D1 to 49-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 49-D1 to 49-D16 each comprise SEQ ID NO:49 as the ST receptor binding moiety.

Compounds 50-D1 to 50-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 50-D1 to 50-D16 each comprise SEQ ID NO:50 as the ST receeptor binding moiety.

Compounds 51-D1 to 51-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 51-D1 to 51-D16 each comprise SEQ ID NO:51 as the ST receptor binding moiety.

Compounds 52-D1 to 52-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 52-D1 to 52-D16 each comprise SEQ ID NO:52 as the ST receptor binding moiety.

Compounds 53-D1 to 53-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 53-D1 to 53-D16 each comprise SEQ ID NO:53 as the ST receptor binding moiety.

Compounds 54-D1 to 54-D16 are the same as compounds 2-D1 to 2-D16, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 54-D1 to 54-D16 each comprise SEQ ID NO:54 as the ST receptor binding moiety.

Compound 2-T1 comprises ricin conjugated to SEQ ID NO:2.

Compound 2-T2 comprises ricin A chain (ricin toxin) conjugated to SEQ ID NO:2.

Compound 2-T3 comprises Pseudomonas cxotoxin (PE) conjugated to SEQ ID NO:2.

Compound 2-T4 comprises diphtheria toxin (DT), conjugated to SEQ ID NO:2.

Compound 2-T5 comprises *Clostridium perfringens* phospholipase C (PLC) conjugated to SEQ ID NO:2.

Compound 2-T6 comprises bovine pancreatic ribonuclease (BPR) conjugated to SEQ ID NO:2.

Compound 2-T7 comprises pokeweed antiviral protein (PAP) conjugated to SEQ ID NO:2.

Compound 2-T8 comprises abrin conjugated to SEQ ID NO:2.

Compound 2-T9 comprises abrin A chain (abrin toxin) conjugated to SEQ ID NO:2.

Compound 2-T10 comprises cobra venom factor (CVF) conjugated to SEQ ID NO:2.

Compound 2-T11 comprises gelonin (GEL) conjugated to SEQ ID NO:2.

Compound 2-T12 comprises saporin (SAP) conjugated to SEQ ID NO:2.

Compound 2-T13 comprises modeccin conjugated to SEQ ID NO:2.

Compound 2-T14 comprises viscumin conjugated to SEQ ID NO:2.

Compound 2-T15 comprises volkensin conjugated to SEQ ID NO:2.

Compounds 3-T1 to 3-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 3-T1 to 3-T15 each comprise SEQ ID NO:3 as the ST receptor binding moiety.

Compounds 5-T1 to 5-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 5-T1 to 5-T15 each comprise SEQ ID NO:5 as the ST receptor binding moiety.

Compounds 6-T1 to 6-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 6-T1 to 6-T15 each comprise SEQ ID NO:6 as the ST receptor binding moiety.

Compounds 7-T1 to 7-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 7-T1 to 7-T15 each comprise SEQ ID NO:7 as the ST receptor binding moiety.

Compounds 8-T1 to 8-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 8-T1 to 8-T15 each comprise SEQ ID NO:8 as the ST receptor binding moiety.

Compounds 9-T1 to 9-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 9-T1 to 9-T15 each comprise SEQ ID NO:9 as the ST receptor binding moiety.

Compounds 10-T1 to 10-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 10-T1 to 10-T15 each comprise SEQ ID NO:2 as the ST receptor binding moiety.

Compounds 11-T1 to 11-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 11-T1 to 11-T15 each comprise SEQ ID NO:11 as the ST receptor binding moiety.

Compounds 12-T1 to 12-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 12-T1 to 12-T15 each comprise SEQ ID NO:12 as the ST receptor binding moiety.

Compounds 13-T1 to 13-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 13-T1 to 13-T15 each comprise SEQ ID NO:13 as the ST receptor binding moiety.

Compounds 14-T1 to 14-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 14-T1 to 14-T15 each comprise SEQ ID NO:14 as the ST receptor binding moiety.

Compounds 15-T1 to 15-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 15-T1 to 15-T15 each comprise SEQ ID NO:15 as the ST receptor binding moiety.

Compounds 15-T1 to 15-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 15-T1 to 15-T15 each comprise SEQ ID NO:15 as the ST receptor binding moiety.

Compounds 17-T1 to 17-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 17-T1 to 17-T15 each comprise SEQ ID NO:17 as the ST receptor binding moiety.

Compounds 18-T1 to 18-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 18-T1 to 18-T15 each comprise SEQ ID NO:18 as the ST receptor binding moiety.

Compounds 19-T1 to 19-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 19-T1 to 19-T15 each comprise SEQ ID NO:19 as the ST receptor binding moiety.

Compounds 20-T1 to 20-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 20-T1 to 20-T15 each comprise SEQ ID NO:20 as the ST receptor binding moiety.

Compounds 21-T1 to 21-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 21-T1 to 21-T15 each comprise SEQ ID NO:21 as the ST receptor binding moiety.

Compounds 22-T1 to 22-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 22-T1 to 22-T15 each comprise SEQ ID NO:22 as the ST receptor binding moiety.

Compounds 23-T1 to 23-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 23-T1 to 23-T15 each comprise SEQ ID NO:23 as the ST receptor binding moiety.

Compounds 24-T1 to 24-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 24-T1 to 24-T15 each comprise SEQ ID NO:24 as the ST receptor binding moiety.

Compounds 25-T1 to 25-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 25-T1 to 25-T15 each comprise SEQ ID NO:25 as the ST receptor binding moiety.

Compounds 26-T1 to 26-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 26-T1 to 26-T15 each comprise SEQ ID NO:26 as the ST receptor binding moiety.

Compounds 27-T1 to 27-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 27-T1 to 27-T15 each comprise SEQ ID NO:27 as the ST receptor binding moiety.

Compounds 28-T1 to 28-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 28-T1 to 28-T15 each comprise SEQ ID NO:28 as the ST receptor binding moiety.

Compounds 29-T1 to 29-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 29-T1 to 29-T15 each comprise SEQ ID NO:29 as the ST receptor binding moiety.

Compounds 30-T1 to 30-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 30-T1 to 30-T15 each comprise SEQ ID NO:30 as the ST receptor binding moiety.

Compounds 31-T1 to 31-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 31-T1 to 31-T15 each comprise SEQ ID NO:31 as the ST receptor binding moiety.

Compounds 32-T1 to 32-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 32-T1 to 32-T15 each comprise SEQ ID NO:32 as the ST receptor binding moiety.

Compounds 33-T1 to 33-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 33-T1 to 33-T15 each comprise SEQ ID NO:33 as the ST receptor binding moiety.

Compounds 34-T1 to 34-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 34-T1 to 34-T15 each comprise SEQ ID NO:34 as the ST receptor binding moiety.

Compounds 35-T1 to 35-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 35-T1 to 35-T15 each comprise SEQ ID NO:35 as the ST receptor binding moiety.

Compounds 36-T1 to 36-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 36-T1 to 36-T15 each comprise SEQ ID NO:36 as the ST receptor binding moiety.

Compounds 37-T1 to 37-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 37-T1 to 37-T15 each comprise SEQ ID NO:37 as the ST receptor binding moiety.

Compounds 38-T1 to 38-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 38-T1 to 38-T15 each comprise SEQ ID NO:38 as the ST receptor binding moiety.

Compounds 39-T1 to 39-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 39-T1 to 39-T15 each comprise SEQ ID NO:39 as the ST receptor binding moiety.

Compounds 40-T1 to 40-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 40-T1 to 40-T15 each comprise SEQ ID NO:40 as the ST receptor binding moiety.

Compounds 41-T1 to 41-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 41-T1 to 41-T15 each comprise SEQ ID NO:41 as the ST receptor binding moiety.

Compounds 42-T1 to 42-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 42-T1 to 42-T15 each comprise SEQ ID NO:42 as the ST receptor binding moiety.

Compounds 43-T1 to 43-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 43-T1 to 43-T15 each comprise SEQ ID NO:43 as the ST receptor binding moiety.

Compounds 44-T1 to 44-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 44-T1 to 44-T15 each comprise SEQ ID NO:44 as the ST receptor binding moiety.

Compounds 45-T1 to 45-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 45-T1 to 45-T15 each comprise SEQ ID NO:45 as the ST receptor binding moiety.

Compounds 46-T1 to 46-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 46-T1 to 46-T15 each comprise SEQ ID NO:46 as the ST receptor binding moiety.

Compounds 47-T1 to 47-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 47-T1 to 47-T15 each comprise SEQ ID NO:47 as the ST receptor binding moiety.

Compounds 48-T1 to 48-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 48-T1 to 48-T15 each comprise SEQ ID NO:48 as the ST receptor binding moiety.

Compounds 49-T1 to 49-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 49-T1 to 49-T15 each comprise SEQ ID NO:49 as the ST receptor binding moiety.

Compounds 50-T1 to 50-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 50-T1 to 50-T15 each comprise SEQ ID NO:50 as the ST receptor binding moiety.

Compounds 51-T1 to 51-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 51-T1 to 51-T15 each comprise SEQ ID NO:51 as the ST receptor binding moiety.

Compounds 52-T1 to 52-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 52-T1 to 52-T15 each comprise SEQ ID NO:52 as the ST receptor binding moiety.

Compounds 53-T1 to 53-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 53-T1 to 53-T15 each comprise SEQ ID NO:53 as the ST receptor binding moiety.

Compounds 54-T1 to 54-T15 are the same as compounds 2-T1 to 2-T15, respectively, except instead of comprising SEQ ID NO:2 as the ST receptor binding moiety, compounds 54-T1 to 54-T15 each comprise SEQ ID NO:54 as the ST receptor binding moiety.

Compounds 2-AP, 3-AP and 5-AP to 54-AP refer to the 51 conjugated compounds that comprise alkaline phosphatase conjugated to SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-NIZ, 3-NIZ and 5-NIZ to 54-NIZ refer to the 51 conjugated compounds that comprise nitroimidazole conjugated to SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-MEZ, 3-MEZ and 5-MEZ to 54-MEZ refer to the 51 conjugated compounds that comprise metronidazole conjugated to SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-MIS, 3-MIS and 5-MIS to 54-MIS refer to the 51 conjugated compounds that comprise misonidazole conjugated to SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-47Sc, 3-47Sc and 5-47Sc to 54-47Sc refer to the 51 conjugated compounds that comprise ^{47}Sc conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-67Cu, 3-67Cu and 5-67Cu to 54-67Cu refer to the 51 conjugated compounds that comprise ^{67}Cu conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-90Y, 3-90Y and 5-90Y to 54-90Y refer to the 51 conjugated compounds that comprise ^{90}Y conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-109Pd, 3-109Pd and 5-109Pd to 54-109Pd refer to the 51 conjugated compounds that comprise ^{109}Pd

conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-123I, 3-123I and 5-123I to 54-123I refer to the 51 conjugated compounds that comprise ^{123}I conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-125I, 3-125I and 5-125I to 54-125I refer to the 51 conjugated compounds that comprise ^{125}I conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-131I, 3-131I and 5-131I to 54-131I refer to the 51 conjugated compounds that comprise ^{131}I conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-132I, 3-132I and 5-132I to 54-132I refer to the 51 conjugated compounds that comprise ^{132}I conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-186Re, 3-186Re and 5-186Re to 54-186Re refer to the 51 conjugated compounds that comprise ^{186}Re, conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-188Re, 3-188Re and 5-188Re to 54-188Re refer to the 51 conjugated compounds that comprise ^{188}Re, conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-199Au, 3-199Au and 5-199Au to 54-199Au refer to the 51 conjugated compounds that comprisc ^{199}Au, conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-211At, 3-211At and 5-211At to 54-211At refer to the 51 conjugated compounds that comprise ^{211}At, conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-212Pb, 3-212Pb and 5-212Pb to 54-212Pb refer to the 51 conjugated compounds that comprise ^{212}Pb conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-212Bi, 3-212Bi and 5-212Bi to 54-212Bi refer to the 51 conjugated compounds that comprise ^{212}Bi conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-203Pb, 3-203Pb and 5-203Pb to 54-203Pb refer to the 51 conjugated compounds that comprise ^{203}Pb conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-206Bi, 3-206Bi and 5-206Bi to 54-206Bi refer to the 51 conjugated compounds that comprise ^{206}Bi conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-32P, 3-32P and 5-32P to 54-32P refer to the 51 conjugated compounds that comprise ^{32}P conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-33P, 3-33P and 5-33P to 54-33P refer to the 51 conjugated compounds that comprise ^{33}P conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-71Ge, 3-71Ge and 5-71Ge to 54-71Ge refer to the 51 conjugated compounds that comprise ^{71}Ge conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-77As, 3-77As and 5-77As to 54-77As refer to the 51 conjugated compounds that comprise ^{77}As conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-103Pd, 3-103Pd and 5-103Pd to 54-103Pd refer to the 51 conjugated compounds that comprisc ^{103}Pd conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-105Rh, 3-105Rh and 5-105Rh to 54-105Rh refer to the 51 conjugated compounds that comprise ^{105}Rh conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-111Ag, 3-111Ag and 5-111Ag to 54-111Ag refer to the 51 conjugated compounds that comprise ^{111}Ag conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-119Sb, 3-119Sb and 5-119Sb to 54-119Sb refer to the 51 conjugated compounds that comprise ^{119}Sb conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-121Sn, 3-121-Sn and 5-121Sn to 54-121Sn refer to the 51 conjugated compounds that comprise ^{121}Sn conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-131Cs, 3-131Cs and 5-131Cs to 54-131Cs refer to the 51 conjugated compounds that comprise ^{131}Cs conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-127Cs, 3-131Cs and 5-131Cs to 54-127Cs refer to the 51 conjugated compounds that comprisc ^{127}Cs conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-129Cs, 3-129Cs and 5-129Cs to 54-129Cs refer to the 51 conjugated compounds that comprise ^{128}Cs conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-143Pr, 3-143Pr and 5-143Pr to 54-143Pr refer to the 51 conjugated compounds that comprise ^{143}Pr conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-161Tb, 3-161Tb and 5-161Tb to 54-161Tb refer to the 51 conjugated compounds that comprise ^{161}Tb conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-177Lu, 3-177Lu and 5-177Lu to 54-177Lu refer to the 51 conjugated compounds that comprise ^{177}Lu conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-191Os, 3-191Os and 5-191Os to 54-191Os refer to the 51 conjugated compounds that comprise ^{191}Os conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-193mPt, 3-193mPt and 5-193mPt to 54-193mPt refer to the 51 conjugated compounds that comprise ^{193M}Pt conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-197Hg, 3-197Hg and 5-197Hg to 54-197Hg refer to the 51 conjugated compounds that comprise ^{197}Hg conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-43K, 3-43K and 5-43K to 54-43K refer to the 51 conjugated compounds that comprise ^{43}K conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-52Fe, 3-52Fe and 5-52Fe to 54-52Fe refer to the 51 conjugated compounds that comprise ^{52}Fe conju-

gated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-57Co, 3-57Co and 5-57Co to 54-57Co refer to the 51 conjugated compounds that comprise ^{57}Co conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-67Ga, 3-67Ga and 5-67Ga to 54-67Ga refer to the 51 conjugated compounds that comprise ^{67}Ga conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-68Ga, 3-68Ga and 5-68Ga to 54-68Ga refer to the 51 conjugated compounds that comprise ^{68}Ga conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-77Br, 3-77Br and 5-77Br to 54-77Br refer to the 51 conjugated compounds that comprise ^{77}Br conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-81Rb, 3-81Rb and 5-81Rb to 54-81Rb refer to the 51 conjugated compounds that comprise ^{81}Rb conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-81mKr, 3-81mKr and 5-81mKr to 54-81mKr refer to the 51 conjugated compounds that comprise ^{81M}Kr conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-87mSr, 3-87mSr and 5-87mSr to 54-87mSr refer to the 51 conjugated compounds that comprise ^{87M}Sr conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-99mTc, 3-99mTc and 5-99mTc to 54-99mTc refer to the 51 conjugated compounds that comprise ^{99M}Tc conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-111In, 3-111In and 5-111In to 54-111In refer to the 51 conjugated compounds that comprise ^{111}In conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

Compounds 2-113mIn, 3-113mIn and 5-113mIn to 54-113mIn refer to the 51 conjugated compounds that comprise ^{113M}In conjugated to SEQ ID NO:2, SEQ ID NO:3 AND SEQ ID NO:5 through SEQ ID NO:54, respectively.

The compounds described in this example are combined with a pharmaceutically acceptable carrier or diluent to produce pharmaceutical compositions according to the present invention. Radiostable compounds described herein are useful in pharmaceutical compositions as therapeutics in the treatment of individuals suspected of suffering from metastasized colorectal cancer including treatment of individuals diagnosed with localized colorectal cancer as a prophylactic/therapeutic before metastasis can be readily detected. When present in therapeutically effective amounts, radioactive compounds described herein are useful in pharmaceutical compositions as therapeutic agents in the treatment of individuals suspected of suffering from metastasized colorectal cancer including treatment of individuals diagnosed with localized colorectal cancer as a prophylactic/therapeutic before metastasis can be readily detected. When present in diagnostically effective amounts, radioactive compounds described herein are useful in pharmaceutical compositions as imaging agents in the diagnosis and identification of metastasized colorectal cancer in individuals.

Example 2

One procedure for crosslinking ST receptor ligands which have a free amino group such as ST receptor binding peptides, as for example SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NOS:5–54 to active agents which bave a free amino group such as methotrexate, doxorubicin, daunorubicin, cytosinarabinoside, cis-platin, vindesine, mitomycin and bleomycin, or alkaline phosphatase, or protein- or peptide-based toxin employs homobifunctional succinimidyl esters, preferably with chain carbon spacers such as disuccinimidyl suberate (Pierce Co, Rockford, Ill.). This approach of amino group derivatization has been employed successfully to crosslink native ST to biotin and, ultimately, to large agarose beads of micron-scale size, preserving the function of native ST (Hughes, M., et al. (1991) *Biochem.* 30:10738; Hakki, S., et al. (1993) *Int. J. Biochem.* 25:557; Almenoff, J. S., et al. (1992) *Mol. Micro.* 8:865; each of which is incorporated herein by reference).

An ST binding ligand with the free amino group such as an ST receptor binding peptide is incubated in the presence of the chemical crosslinking agent and an active agent which have a free amino group in equimolar quantities at room temperature for 15–30 min. Incubation is terminated by separating the reactants by gel permeation chromatography by HPLC. This technique separates the conjugated compounds from free active agents and free ST binding ligands, active agent-active agent conjugates and ST binding ligand-ST binding ligand conjugates. Homogeneous preparations of conjugated through their free amino groups and with a preferred molar ratio of 1:1 are obtained. As indicated above, complexing the free amino group of an ST peptide preserves receptor binding function.

Example 3

In the event that a cleavable conjugated compound is required, the same protocol as described above may be employed utilizing 3,3'-dithiobis (sulfosuccinimidylpropionate (SPDP); Pierce, Ill.). SPDP forms a sulfhydral group from a free amino group which may be used to conjugate a compound to another free amino group. For example, ST peptides such as SEQ ID NO:2, SEQ ID NO:3, SEQ ID NOS:5–54 are derivatized using established procedures employing N-succinimidyl-3 (2-pyridildithio)propionate (SPDP, Pharmacia-LKB, New Jersey). The ST peptide is incubated with a 5-fold molar excess of SPDP for 30 minutes at room temperature. The ST-pyridylthiopropionate conjugate is separated from unreacted reagents by gel permeation chromatography by HPLC. An active agent with a free amino group, such as a protein-based toxin, is prepared for conjugation by reduction with dithiothreitol for 4 hours at room temperature. Reduced active agent is incubated with a 2-fold molar excess of ST receptor ligand-PDP conjugate at pH 8.0 for 36 hours at 4°C. Conjugate compound is purified from unreacted agents by gel permeation chromatography by HPLC.

This protocol for conjugation is particularly useful to conjugate ST peptides to diphtheria toxin A chains and Pseudomonas exotoxin as well as ricin toxin A chains (Magerstadt, M. *Antibody Conjugates and Malignant Disease.* (1991) CRC Press, Boca Raton, USA, pp. 110–152; Cawley, D. B. et al. (1980) Cell 22:563; Cumber, A. J., et al. (1985) *Meth. Enz.* 112:207; Gros, O. (1985) *J. Immunol. Meth.* 81:283; Worrell, N. R., et al. (1986) *Anti-Cancer Drug Design* 1:179; Thorpe, P. E. et al. (1987) *Cancer Res.* 47:5924, each of which is incorporated herein by reference).

Example 4

Active agents with a free amino group may be derivatized with SPDP as described above and conjugated with an ST

ligand that has a free amino group and that has been modified with the succinimidyl ester of iodoacetic acid (Pierce Co., Rockford, Ill.) (Magerstadt, M. (1991) *Antibody Conjugates And Malignant Disease*, CRC Press Boca Raton; Cumber, A. J. et al. (1985) *Meth. Enz.* 112:20, which are incorporated herein by reference). Conjugation relies on the selective reaction of iodoacctyl groups introduced into the amino terminal of the ST ligand with the thiol groups introduced into the active agent. As with the above protocol, this procedure avoids homopolymer formation. However, the product is conjugated through a central thioether linkage which cannot be reduced.

Example 5

An ST receptor ligand with a free amino group and active agents with free amino groups may be conjugated through a disulfide bond using iminothiolane (Pierce, Rockford, Ill.) (Fitzgerald, D. J. P. et al. (1983) *Cell* 32:607; Magerstadt, M. (1991) *Antibody Conjugates And Malignant Disease*, CRC Press, Boca Raton; Björn, M. J., et al. (1985) *Cancer Res.* 45:1214; Björn, M. J., et al. (1986) *Cancer Res.* 46:3262, which are incorporated herein by reference). The ST receptor ligand with a free amino group is derivatized at the amino terminal with iminothiolane and the active agent is derivatized with SPDP as described above. Reacting iminothiolane-derivatized ST receptor ligand with SPDP-derivatized active agent results in conjugation by a reducible disulfide bond. In addition, iminothiolane provides the versatility to conjugate these proteins through bonds other than disulfides. Thus, derivatization of active agents with the heterobifunctional agent sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane (Pierce, Rockford, IL) and reaction with iminothiolane-derivatized ST receptor ligand will conjugate these peptides without formation of disulfides.

Example 6

Conjugated compounds according to the invention which comprise an active moiety that is a therapeutic agent specifically inhibit T84 cells in vitro. The following protocols may be used to demonstrate that the conjugated compounds according to the invention which comprise an active moiety that is chemotherapeutic or toxin specifically inhibit T84 cells in vitro. Inhibition of T84 cells is assessed by determining the effects of conjugated compounds on the ability of T84 cells to incorporate ^{35}S-leucine into protein, ^{3}H-thymidine into DNA, and to form colonies. The assessment of protein and DNA synthesis are classical techniques to determine the cytotoxicity of conjugated compounds in vitro. Inhibition of protein synthesis is measured because the toxins used as active moieties are specific inhibitors of this process. Therefore, these assays are the most sensitive measure of whether conjugated compounds are binding to and internalized into T84 cells. Inhibition of DNA synthesis is measured because some chemotherapeutics inhibit DNA synthesis and further, it is a cytotoxicity assay which correlates closely with the reproductive survivability of cells in culture. Cytotoxicity, or the disruption of normal cellular metabolic processes, may not always directly correlate with cell survivability. Therefore, assessment of colony formation will directly measure the ability of the experimental agents to decrease the survivability of tumor cells, which closely correlates with the impact of therapeutic agents on tumor viability in vivo. Controls include performing the same assay using the unconjugated form of the active agent and the unconjugated form of the ST receptor ligand of which the conjugated compound is comprised in place of the conjugated compound. The results obtained in the test assays and control assays are compared.

Conjugated compounds are assessed for their ability to inhibit protein and DNA synthesis in vitro and to inhibit survival and proliferation by measuring colony formation in monolayer culture by established protocols (Wilson, A. P. (1987) "Cytotoxicity and viability assays", Animal Cell Culture: A Practical Approach. Freshney, R. I., ed. pp. 183–216, IRL Press, Oxford. which is incorporated herein by reference).

To assess the ability of a conjugated compound to inhibit protein synthesis in vitro, cells are plated in 200 μl of medium at a sub-confluent density of $1–2\times10^5$ and allowed to attach to form a dividing cell monolayer over 12 hours at 37° C. Subsequently, the media is replaced with 200 μl of fresh media containing the appropriate concentration of conjugated compounds and cells incubated at 37° C. for various amounts of time. At the end of the indicated incubation period, cells is washed twice with medium and incubated at 37° C. in 0.5 ml of methionine-free medium supplemented with 0.5 μCi of L^{35}S-methionine (800 Ci/mmol). After incubation for another 2 hours at 37° C., the medium is aspirated, cells washed twice with medium containing 1 mg/ml of methionine, and then precipitated in 12% ice-cold TCA. Radioactivity recovered in TCA precipitates by centrifugation is quantified by liquid scintillation spectroscopy. In these studies, cells are maintained in log growth and assays are performed using triplicate wells. Data is expressed as a percentage of protein synthesis observed in the presence of experimental agents compared to untreated cells.

To assess the ability of a conjugated compound to inhibit DNA synthesis in vitro cells are plated as a subconfluent monolayer and incubated with experimental agents as described above. At the end of the incubation period, cells are washed twice and incubated at 37° C. in medium containing 2.5 μCi of ^{3}H-thymidine (5 Ci/mmol). After incubation for another hour, cells are processed with TCA, precipitates recovered, and radioactivity quantified as described above. As above, cells are maintained in log growth and assays is performed in triplicate. Data is expressed as a percentage of DNA synthesis observed in the presence of experimental agents compared to untreated cells.

To assess the ability of a conjugated compound to inhibit survival and proliferation by measuring colony formation in monolayer culture, cells are plated as a sub-confluent monolayer on 25 cm^2 flasks and allowed to attach as described above. The medium is replaced with that containing various concentrations of experimental agents and incubated with cells for various amounts of time. At the end of the incubation, cells are recovered as a single cell suspension by trypsinization and replated to a density which will yield 100– 200 colonies per 6 cm plate. Cells are permitted to grow for 7 days, then fixed in methanol, stained with 1% crystal violet, and the number of colonies quantified. Assays are performed in duplicate and data is expressed as a percentage of colony formation observed in the presence of experimental agents compared to untreated cells. Results in our laboratory have demonstrated that T84 cells can be placed into single cell suspensions utilizing trypsin (10 μg/ml) with a plating efficiency of 40% and a doubling time of 18 hours.

Example 7

Radioactive iodine such as ^{123}I, ^{125}I, ^{131}I and ^{132}I, can be added to an ST receptor binding peptide such as an ST

peptide using a standard protocol well-known to those having ordinary skill in the art (Thompson, M. et al. (1985) *Analytical Biochemistry* 148:26, which is incorporated herein by reference). Radioactive iodine is conjugated directly to an ST peptide such as SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:5 at tyrosine-5, tyrosine-4 or tyrosine-5, respectively.

Briefly, the ST peptide is produced in bacteria. For example, *E. coli* strain 431 is grown in culture and secretes ST into this culture. The culture media is then purified using routine techniques. ST can also be made by solid-phase synthesis as has been done previously, using standard techniques. (Dreyfus, L., et al. (1983) *Infec. Immun.* 42:539, which is incorporated herein by reference.

Ten micrograms of ST peptide are reacted with 2 milliCuries of radioactive INa (Amersham Corporation, Massachusetts) in the presence of Iodobeads (Bio Rad Laboratorics, California) and beta-D-glucose. These are reacted for 30 min after which the products are subjected to chromatography on a Sepak reversed-phase cartridge (Millipore Corp., Massachusetts) followed by separation on a C_{18}reversed-phase column by HPLC using a 20– 25% acetonitrile gradient. Conjugated compositions which comprise SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:5 with the radioiodine attached to tyrosine-4 elutes at 45 min. These molecules retain full biochemical and pharmacological activity.

Example 8

^{125}I is conjugated directly to an ST peptide such as SEQ ID NO:13 at tyrosine-4.

SEQ ID NO:13 is produced by solid-phase synthesis as described above. Ten micrograms of SEQ ID NO:13 are reacted with 2 milliCuries of ^{125}INa (Amersham Corporation, Massachusetts) in the presence of Iodobeads (Bio Rad Laboratories, California) and beta-D-glucose. These are reacted for 30 min after which the products are subjected to chromatography on a Sepak reversed-phase cartridge (Millipore Corp., Massachusetts) followed by separation on a C_{18}reversed-phase column by HPLC using a 20– 25% acetonitrile gradient. ^{125}I-SEQ ID NO:13 conjugate with the radioiodine attached to tyrosine-4 elutes at 45 min. This molecule retains full biochemical and pharmacological activity.

Dosing of radioiodine for diagnostic imaging typically requires about 4 milliCuries/patient (Steinstraber, A., et al. (1988) *J. Nucl. Med.* 29:875; Wessels, B. W. and Rogus, R. D. (1984) *Med. Phys.* 11:638; Kwok, C. S., et al. (1985) *Med. Phys.* 12:405). For proteins labeled with a specific activity of 2,000 Curies/mmol, such as ST peptide, this would require about 10 micrograms of labeled peptide injected intravenously per patient for diagnostic imaging.

Example 9

^{131}I is conjugated directly to an ST peptide such as SEQ ID NO:13 at tyrosine-4.

SEQ ID NO:13 is produced by solid-phase synthesis as described above. Ten micrograms of SEQ ID NO:13 are reacted with 10 milliCuries of ^{131}INa (Amersham Corporation, Massachusetts) in the presence of Iodobeads (Bio Rad Laboratories, California) and beta-D-glucose. These are reacted for 30 min after which the products are subjected to chromatography on a Sepak reversed-phase cartridge (Millipore Corp., Massachusetts) followed by separation on a C_{18}reversed-phase column by HPLC using a 20– 25% acetonitrile gradient. ^{131}I-SEQ ID NO:13 conjugate with the radioiodine attached to tyrosine-4 elutes at 45 min. This molecule retains full biochemical and pharmacological activity.

Typically, for radioiodinated antibodies (MW=160,000 Da), about 150 nanomoles of protein (24 milligrams) labeled with a specific activity of 10,000 Curics/mmol are required per gram of tumor per patient (Humm, J. L. (1986) *J. Nucl. Med.* 27:1490). Thus, for proteins labeled with a specific activity of 2,000 Curies/mmol, with a molecular weight of 2,000 Da, such as ST peptide, about 3 milligrams would be required per gram of tumor per patient for intravenous infusion.

Example 10

In some embodiments, coupling of ST receptor ligands which have a free amino group, particularly ST receptor binding peptides such as ST peptides, and active agents with a free amino group such as protein-based toxins is performed by introducing a disulfide bridge between the 2 molecules. This strategy is particularly useful to conjugate ST peptides since the free amino terminal has been shown to be useful as a point of conjugation without affecting ST binding activity. This strategy is particularly useful to conjugate protein-based toxins since the free amino terminal is available on such molecules and for some conjugated compounds, most notably RTA conjugates, a disulfide bride which can be reduced to yield separate proteins has been demonstrated to be important in the construction of functional chimeras targeted by monoclonal antibodies (Magerstadt, M. (1991) *Antibody Conjugates And Malignant Disease*, CRC Press, Boca Raton; Bjorn, M. J., et al. (1985) *Cancer Res.* 45:1214; Bjorn, M. J., et al. (1986) *Cancer Res.* 46:3262; Masuho, Y., et al. (1982) *J. Biochem.* 91:1583, which are each incorporated herein by reference). While some toxins may be coupled to ST peptides using crosslinking agents which do not result in a reducible disulfide bridge between the individual components but retain functional cytotoxicity, ricin A chain toxin requires a reducible disulfide for cytotoxicity while Pseudomonas exotoxin, for example, does not.

Disulfide coupling is achieved using established procedures employing the heterobifunctional agent N-succinimidyl-3 (2-pyridyldithio)-proportionate (SPDP, Pharmacia-LKB, Piscataway, N.J.) (Magerstadt, M. (1991) *Antibody Conjugates And Malignant Disease*, CRC Press, Boca Raton; Cawley, D. B. et al. (1980) *Cell* 22:563; Cumber, A. J., et al. (1985) *Meth. Enz.* 112:20; Gros, O., et al. (1985) *J. Immunol. Meth.* 81:283; Worrell, N. R., (1986) *Anti-Cancer Drug Design* 1:19; Thorpe, P. E., et al. (1987) *Cancer Res.* 4:5924, which are incorporated herein by reference).

In some embodiments, toxins including the A chains of deglycosylated ricin toxin (RTA; Sigma Chemical Co., St. Louis, Mo.), diphtheria toxin A (DTA; Calbiochem, La Jolla, Calif.) and Pseudomonas exotoxin (PEA) are conjugated to ST peptides to produce conjugated compositions according to the present invention using this procedure. Deglycosylated RTA is employed since the glycosylated form of this toxin exhibits non-specific binding to liver cells. DTA is prepared from diphtheria toxin by an established procedure (Michel, A. and Drykx, J. (1975) *Biochem. Biophys. Acta* 365:15; Cumber, A. J., et al. (1985) *Meth. Enz.* 112:207, both of which are incorporated herein by reference). PEA

In some embodiments, ST peptides are conjugated to toxins by this procedure. For example, the ST peptide SEQ ID NO:3 which is produced as described above (see Drey-

fus, L., et al. (1983) *Infec. Immun.* 42:539, which is incorporated herein by reference).

Toxins are prepared for coupling by reduction with 0.1M dithiothreitol (DTT) for 4 hours at room temperature in 0.4M Tris-HCl, pH 8.0 and 1 mM EDTA. Reduced toxins are desalted on a Sephadex G-25 column equilibrated in TES buffer and mixed with a 2-fold molar excess of ST-PDP. Reactions are adjusted to pH 8.0 with TES and incubated at 4° C. for 36 hours. ST peptide-toxin conjugates are purified from unreacted products and homopolymers of ST peptides and toxins by gel filtration on Sepbadex G-75 in 20 mM TES, pH 8.0 containing 0.1M NaCl. Chromatographic fractions are monitored by SDS-PAGE on 10% polyacrylamide gels under non-reducing conditions for the presence of 1:1 conjugates of ST peptides and toxins. Also, these conjugates are analyzed by 10% SDS-PAGE under reducing conditions, to insure that ST and cytotoxins are coupled by a reducible disulfide bond. Molar concentrations of the conjugate are calculated by quantifying radioactivity in these samples.

ST trace labeled with ^{125}I on tyrosine 4 (10 Ci/mmol) is used in order to follow the conjugate through various separation and chromatographic steps and to enable us to calculate the molar ratio of ST to cytotoxin in the final purified conjugate. ST trace labeled with ^{125}I is derivatized by incubating 1 mg/ml with a 5-fold molar excess of SPDP for 30 min at room temperature in Na phosphate buffer, pH 7.4. The ST-pyridylthiopropionate (ST-PDP) conjugate is purified from unreacted crosslinking agent by chromatography on Sephadex G-25 equilibrated with 20 mM N-Tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES) buffer, pH 7.4. Preservation of receptor binding of conjugated ST peptides in human intestinal membranes is determined in competition assays of increasing concentrations of ST-PDP and ^{125}I-ST (5×10^{10}M), to insure that this process does not destroy the function of the ST receptor ligand.

The above coupling protocol has several advantages for conjugating the various toxins. First, it introduces a reducible disulfide bridge into the conjugated composition, important for RTA cytotoxicity. Also, this technique avoids the exposure of ST peptide to quantitative reduction with DTT which could interrupt its 3 intracbain disulfide bonds important for receptor binding activity. In addition, there is a single group available at the amino terminal of ST peptide for derivatization with SPDP and previous experiments have demonstrated that derivatization of that group preserves the binding properties of the ligand. Therefore, other configurations for conjugation which could result in inactivation of ST are not possible. Furthermore, PEA requires preactivation with DTT to achieve optimum cytotoxicity which will be accomplished utilizing the above protocol.

To produce a functional conjugated compound that comprises a toxin, it is essential that the receptor binding and enzyme activities of the moieties are preserved throughout the process of conjugation. Therefore, once such conjugate compounds are obtained, they are tested for the preservation of those functions. ST receptor binding activity of conjugated compounds is examined in competitive binding assays, as described above. In these studies, increasing concentrations of the conjugated compounds are incubated with a constant concentration (5×10^{10}M) of ^{125}I-ST and intestinal membranes (50–100 μg of protein) to achieve equilibrium. Parallel incubations contain excess (5×10^{7}M) unlabeled ST to assess non-specific binding. The concentration-dependent competitive displacement of radiolabeled ST by conjugated compounds is compared to the competitive displacement achieved by native ST. Displacement curves are employed to estimate the affinity of each conjugated compound (K_D) and compare that to the affinity of native ST measured by this technique. Control studies include evaluating the ability of unconjugated toxins to compete with native ST for receptor binding. These studies establish that the binding function of ST in the conjugated construct is preserved.

Preservation of toxin activity in conjugated compounds is also assessed. PEA and DTA induce toxicity by catalyzing the NAD-dependent ADP-ribosylation of elongation factor 2 (EF2), inhibiting protein synthesis. ADP-ribosyl transferase activity is assessed using an established assay (Chung, D. W. and Collier, R. J. *Infect. Immun.* 16:832; Fitzgerald, D. J. P. (1987) *Meth. Enz.* 151:139, which are both incorporated herein by reference). Reactions are conducted in 30 Mm Tris-HCl, pH 8.2 containing 40 mM DTT, 50 mCi ^{14}C-NAD, and 20 μl of rabbit reticulocyte lysate containing elongation factor 2 (EF-2; Promega, Madison, Wis.) in a total volume of 500 μl. Reactions are initiated by the addition of lysate, incubated for 30 minutes at 37° C., and terminated by the addition of ice-cold 12% TCA. Radioactivity in protein precipitates collected by centrifugation is quantified by liquid scintillation spectroscopy. The ability of the conjugated compounds that comprise DTA or PEA to catalyze the transfer of labeled ADP-ribose to EF-2 is compared to that catalyzed by similar quantities of unconjugated toxins. Control experiments include examining the ability of unconjugated toxins or ST to catalyze ADP-ribose transfer and the effects of ST on the enzymatic activity of unconjugated cytotoxins.

RTA inhibits protein synthesis by catalytically inactivating the 608 ribosomal subunit. The catalytic activity of conjugated compounds that comprise RTA is assessed by its ability to inhibit protein synthesis in cell-free assays using established procedures (Leonard, J. E. et al. (1985) *Cancer Res.* 45:5263 which is incorporated herein by reference). Assays contain 35 μl of nuclease-treated rabbit reticulocyte lysates, 1 μl of 1 mM mixed amino acids deficient in methionine, 2 μl of Brome mosaic RNA (Promega, Madison, Wis.) at 0.5 μg/μl, 7 μl of sterile water or conjugate solution, and 5 μCi of ^{35}S-methionine in a total volume of 50 μl. Reactions will be initiated by the addition of lysate, incubated at 30° C. for 30 minutes, and terminated by the use of addition of 12% TCA. Radioactivity in protein precipitates collected by centrifugation is quantified by liquid scintillation spectroscopy. Control experiments include examining the ability of unconjugated RTA or ST peptide to inhibit cell-free protein synthesis and the effects of ST peptide on the inhibitory activity of the unconjugated cytotoxin.

Example 11

Methotrexate is linked to SEQ ID NO:12 by the homobifunctional crosslinker succinimidyl esters with long chain carbon spacers such as disuccinimidyl suberate (Pierce, Ill.). SEQ ID NO:12 is incubated in the presence of the chemical crosslinking agent and methotrexate in equimolar quantities at room temperature for 15–30 min. Incubation is terminated by separating the reactants by gel permeation chromatography by HPLC. This technique separates the methotrexate/SEQ ID NO:12 conjugates from free drug, free ST peptide, drug-drug conjugates and ST peptide-ST peptide conjugates. Homogeneous preparations of SEQ ID NO:12-methotrexate conjugates coupled through their free amino groups and with a preferred molar ratio of 1:1 are obtained. Complexing the free amino group of ST preserves receptor binding function.

Example 12

^{111}In is coupled to SEQ ID NO:37 with functional amino groups using a chelator. The ST peptide has a free amino function at the amino terminal which may be modified without altering the ST receptor binding activity of the ST peptide. ^{111}In is rapidly and potently chelated by either EDTA (ethylenediaminetetraacetic acid) or DTPA (diethylenetriaminepetaacetic acid). DTPA is preferred over EDTA because the latter may be more unstable in vivo. The ^{111}In-DTPA is converted to a mixed N-hydroxysuccinimide ester which is reactive with free amino groups, mixed with ST, and the reaction products, including ^{111}In-SEQ ID NO:37 separated by HPLC (Bremer, K. H. and Schwarz, A. (1987) in *Safety And Efficacy Of Radiopharmaceuticals*. Kristensen, K. and Norbygaard, E., Eds. Martinius Nijboff, Dordrecht, The Netherlands, P. 43; Krejcarek, G. E., and Tucker, K. L. (1977) *Biochem. Biophys. Res. Commun.* 77:581; Paxton, R. J., et al. (1985) *Cancer Res.* 45:5694; Richardson, A. P., et al. (1986) *Nucl. Med. Biol.* 14:569, which are each incorporated herein by reference).

Example 13

^{99m}Tc can be conjugated to SEQ ID NO:46 using an approach which is similar to that for indium. Thus, technetium can be chelated by DTPA which is converted to an anhydride, such as N-hydroxysuccinimide anhydride, and reacted with SEQ ID NO:46. The ST-technetium conjugate can then be separated using HPLC (Magerstadt, M. (1991) *Antibody Conjugates And Malignant Disease* CRC Press, Boca Raton; Eckelman, W. C. and Paik, C. H. (1986) *Nucl. Med. Biol.* 14:569)

Example 14

Diphtheria toxin A chain (DTA) is prepared from native diphtheria toxin by standard techniques. SEQ ID NO:22 is coupled to N-succinimidyl-3(2-pyridyldithio)-propionate (SPDP, Pharmacia-LKB, Piscataway, N.J.) and the SEQ ID NO:22-PDP conjugate is purified by HPLC by established procedures. DTA is reduced with dithiothreitol and incubated with SEQ ID NO:22-PDP. DTA-SEQ ID NO:22 is purified after conjugation using HPLC.

Example 15

Pseudomonas Exotoxin is prepared from native sources by standard techniques. SEQ ID NO:54 is coupled to N-succinimidyl-3(2-pyridyldithio)-propionate (SPDP, Pharmacia-LKB, Piscataway, N.J.) and the SEQ ID NO:54-PDP conjugate is purified by HPLC by established procedures. Pseudomonas Exotoxin is reduced with dithiothreitol and incubated with SEQ ID NO:54-PDP. Pseudomonas Exotoxin-SEQ ID NO:54 is purified after conjugation using HPLC.

Example 16

Doxorubicin is linked to SEQ ID NO:54 by the homobifunctional crosslinker succinimidyl esters with long chain carbon spacers such as disuccinimidyl suberate (Pierce, Ill.). SEQ ID NO:54 is incubated in the presence of the chemical crosslinking agent and doxorubicin in equimolar quantities at room temperature for 15–30 min. Incubation is terminated by separating the reactants by gel permeation chromatography by HPLC. This technique separates the doxorubicin/SEQ ID NO:54 conjugates from free doxorubicin, free ST peptide, drug-drug conjugates and ST peptide-ST peptide conjugates. Homogeneous preparations of SEQ ID NO:54-doxorubicin conjugates coupled through their free amino groups and with a preferred molar ratio of 1:1 are obtained. Complexing the free amino group of ST preserves receptor binding function.

Example 17

Daunorubicin is linked to SEQ ID NO:32 by the homobifunctional crosslinker succinimidyl esters with long chain carbon spacers such as disuccinimidyl suberate (Pierce, Ill.). SEQ ID NO:32 is incubated in the presence of the chemical crosslinking agent and daunorubicin in equimolar quantities at room temperature for 15–30 min. Incubation is terminated by separating the reactants by gel permeation chromatography by HPLC. This technique separates the daunorubicin/SEQ ID NO:54 conjugates from free daunorubicin, free ST peptide, drug-drug conjugates and ST peptide-ST peptide conjugates. Homogeneous preparations of SEQ ID NO:54-daunorubicin conjugates coupled through their free amino groups and with a preferred molar ratio of 1:1 are obtained. Complexing the free amino group of ST preserves receptor binding function.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 54

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:

-continued

(A) NAME/KEY: CDS
(B) LOCATION: 1..57

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
AAC AAC ACA TTT TAC TGC TGT GAA CTT TGT TGT AAT CCT GCC TGT GCT    48
Asn Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala
 1               5                  10                  15

GGA TGT TAT                                                      57
Gly Cys Tyr
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Asn Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala
 1               5                  10                  15

Gly Cys Tyr
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Ala Gly
 1               5                  10                  15

Cys Asn
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..57

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
AAT AGT AGC AAT TAC TGC TGT GAA TTG TGT TGT AAT CCT GCT TGT AAC    48
Asn Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Asn
 1               5                  10                  15

GGG TGC TAT                                                      57
Gly Cys Tyr
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Asn Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Asn
  1               5                  10                  15

Gly Cys Tyr
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Pro Asn Thr Cys Glu Ile Cys Ala Tyr Ala Ala Cys Thr Gly Cys
  1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Asn Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala
  1               5                  10                  15

Gly Cys
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly
  1               5                  10                  15

Cys
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Thr Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys
  1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys
 1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly
 1               5                  10                  15

Cys Tyr
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Thr Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys
 1               5                  10                  15

Tyr
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys Tyr
 1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys Tyr
 1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys Tyr
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Ala Gly
 1               5                  10                  15

Cys
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
Thr Phe Tyr Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Ala Gly Cys
 1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
Phe Tyr Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Ala Gly Cys
```

-continued

1 5 10 15

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Tyr Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Ala Gly Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Ala Gly Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Thr Phe Tyr Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Ala Gly Cys
1 5 10 15

Asn

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Phe Tyr Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Ala Gly Cys Asn
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Tyr Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Ala Gly Cys Asn
1 5 10 15

-continued

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Ala Gly Cys Asn
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
Asn Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr
 1               5                  10                  15

Gly Cys
```

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```
Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly
 1               5                  10                  15

Cys
```

(2) INFORMATION FOR SEQ ID NO:29.

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```
Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys
 1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS.
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```
Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys
 1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```
Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```
Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly
 1               5                  10                  15

Cys Tyr
```

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION. SEQ TD NO:34:

```
Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys
 1               5                  10                  15

Tyr
```

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:35:

```
Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
 1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:36:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```
Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
 1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:38:

```
Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly
 1               5                  10                  15

Cys Tyr
```

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:39:

```
Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Ala Pro Ala Cys Ala Gly
 1               5                  10                  15

Cys Tyr
```

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:40:

```
Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Asn Ala Ala Cys Ala Gly
 1               5                  10                  15

Cys Tyr
```

(2) INFORMATION FOR SEQ ID NO:41:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:41:

```
Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly
 1               5                  10                  15

Cys
```

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:42:

```
Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys Tyr
 1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```
Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys Tyr
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:45:

```
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids

-continued

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:46:

```
Gln Ala Cys Asp Pro Pro Ser Pro Pro Ala Glu Val Cys Cys Asp Val
 1               5                  10                  15

Cys Cys Asn Pro Ala Cys Ala Gly Cys
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:47:

```
Ile Asp Cys Cys Ile Cys Cys Asn Pro Ala Cys Phe Gly Cys Leu Asn
 1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:48:

```
Ser Ser Asp Trp Asp Cys Cys Asp Val Cys Cys Asn Pro Ala Cys Ala
 1               5                  10                  15

Gly Cys
```

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:49:

```
Asn Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Tyr Pro Ala Cys Thr
 1               5                  10                  15

Gly Cys Tyr
```

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:50:

```
Cys Cys Asp Val Cys Cys Asn Pro Ala Cys Thr Gly Cys
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:51:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:51:

```
Cys Cys Asp Val Cys Cys Tyr Pro Ala Cys Thr Gly Cys Tyr
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:52:

```
Cys Cys Asp Leu Cys Cys Asn Pro Ala Cys Ala Gly Cys Tyr
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:53:

```
Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr Gly Cys Tyr
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:54:

```
Pro Gly Thr Cys Glu Ile Cys Ala Tyr Ala Ala Cys Thr Gly Cys
 1               5                  10                  15
```

I claim:

1. A method of imaging metastasized colorectal cancer cells in an individual comprising the steps of:

a) administering into the circulatory system of said individual, a diagnostically effective amount of a pharmaceutical composition comprising:

i) a pharmaceutically acceptable carrier or diluent, and,

ii) a conjugated compound comprising:

1) a ST receptor binding moiety; and,

2) an active moiety;

wherein said ST receptor binding moicty is a heat stable (ST) toxin peptide having less than 25 amino acids or fragments or derivatives thereof, wherein said heat stable toxin, fragments or derivatives thereof specifically bind to the ST receptor, and said active moiety is an imaging agent which can be detected in said individual's body; and

b) detecting localization and accumulation of said imaging agent in said individual's body.

2. The method of claim **1** wherein said pharmaceutical composition is administered to said individual intravenously.

3. A method of claim **1** wherein said imaging agent is radioactive.

4. The method of claim **3** wherein said ST receptor binding moiety has 13-25 amino acids.

5. The method of claim **3** wherein said ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:54.

6. The method of claim **3** wherein said active moiety is selected from the group consisting of: ^{43}K, ^{52}Fe, ^{57}Co, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{77}Br, $^{81}Rb/^{81M}Kr$, ^{87M}Sr, ^{99M}Tc, ^{111}In, ^{113M}In, ^{123}I, ^{125}I, ^{127}Cs, ^{129}Cs, ^{131}I, ^{132}I, ^{197}Hg, ^{203}Pb and ^{206}Bi.

7. The method of claim **3** wherein said active moiety is selected from the group consisting of: ^{99M}Tc, ^{111}In and ^{125}I.

8. The method of claim 3 wherein said active moiety is selected from the group consisting of: radioactive iodine and radioactive indium.

9. The method of claim 3 wherein said ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:54.

10. The method of claim 3 wherein said

ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:54; and

said active moiety is selected from the group consisting of: ^{43}K, ^{52}Fe, ^{57}Co, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{77}Br, $^{81}Rb/^{81m}Kr$, ^{87m}Sr, ^{99m}Tc, ^{111}In, ^{113m}In, ^{123}I, ^{125}I, ^{127}Cs, ^{129}Cs, ^{131}I, ^{132}I, ^{197}Hg, ^{203}Pb and ^{206}Bi.

11. The method of claim 3 wherein said

ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:54; and

said active moiety is selected from the group consisting of: ^{99m}Tc, ^{111}In and ^{125}I.

12. The method of claim 3 wherein said pharmaceutical composition is administered in a dose of 0.1–100 millicuries.

13. The method of claim 3 wherein said pharmaceutical composition is administered in a dose of 1–10 millicuries.

14. The method of claim 3 wherein said pharmaceutical composition is administered in a dose of 2–5 millicuries.

15. The method of claim 14 wherein said conjugated compound consists of ^{125}I linked to SEQ ID NO:13.

16. The method of claim 3 wherein said conjugated compound consists of ^{111}In linked to SEQ ID NO:37.

17. The method of claim 3 wherein said conjugated compound consists of ^{99m}Tc linked to SEQ ID NO:46.

18. A method of imaging metastasized colorectal cancer cells in an individual comprising the steps of:

a) administering into said individual's circulatory system, a diagnostically effective amount of a pharmaceutical composition comprising:

i) a pharmaceutically acceptable carrier or diluent, and,

ii) a conjugated compound comprising:

1) a ST receptor binding moiety; and,

2) an active moiety;

wherein said ST receptor binding moiety is a heat stable (ST) toxin peptide having less than 25 amino acids or fragments or derivatives thereof, wherein said heat stable toxin, fragments or derivatives thereof specifically bind to the ST receptor, and said active moiety is an imaging agent which can be detected in said individual's body by radioscintigraphy, nuclear magnetic resonance imaging, computed tomography or positron emission tomography; and

b) detecting localization and accumulation of said imaging agent in said individual's body by radioscintigraphy, nuclear magnetic resonance imaging, computed tomography or positron emission tomography.

19. The method of claim 18 wherein said pharmaceutical composition is administered to said individual intravenously.

20. The method of claim 18 wherein said ST receptor binding moiety has 13–25 amino acids.

21. The method of claim 18 wherein said ST receptor binding moiety is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:54.

22. A method of claim 18 wherein said imaging agent is detected by radioscintigraphy.

* * * * *